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Immunodiagnosis of Fasciolosis by Detection of Coproantigen.

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IMMUNODIAGNOSIS OF FASCIOLOSIS BY DETECTION OF COPROANTIGEN

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LIST OF ABBREVIATIONS

CAA:	Circulating anodic antigen
CCA:	Circulating cathodic antigen
CEP:	Counterelectrophoresis
CF:	Complement fixation test
DTAF:	Dichlorotriazinyle amino flourescien
ECL:	Enhanced chemiluminescence substrate
ELISA:	Enzyme-linked immunosorbent assay
Endo F:	Endoglycosidase F
Endo H:	Endoglycosidase H
ES:	Excretory secretory product
FFA:	Fresh fluke antigen
IFA:	Indirect fluorescence assay
Kd	Kilo-Dalton
Mab:	Monoclonal antibodies
Mabs:	Monoclonal antibodies
MAb F10:	M2DS/DSF10
MAb G7:	M7 D5/G7
MAb E7:	M1CS/E7
MAb F7:	M7D5/A11F7
2 ME:	2 - mercaptoethanol
MW	Relative molecular weight
NET:	0.05M Tris, 0.001M EDETA and 0.15 NaCl pH 7.4
NET-T:	0.05M Tris, 0.001M EDETA and 0.15 NaCl pH 7.4 containing 0.1% Tween 20
OD:	Optical density
PAS:	Periodic acid Schiff reagent
PBS:	0.01 M phosphate buffered saline, pH 7.2
SDS-PAGE:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TMB:	3.3',5,5' tetra methylebenzidine
WB:	Western blot analysis

ABSTRACT

A monoclonal antibody (MAb)-based western blot analysis (WB) was developed for detection of a 26-28 kD coproantigen in *Fasciola. hepatica* infected cattle. Using WB the coproantigen was detected in feces from experimentally infected calves with 22 flukes or more. Using the biotin-streptavidin modification of WB, antigen was detected in the feces from experimentally-infected calves with 10 flukes or more as early as 6 weeks post-infection. The MAbs did not react with the ES of *Paramphistomum* sp. or *Moniezia* sp, but showed slight cross-reactivity with ES from *F. gigantica*, and there was moderate cross-reactivity with *Fascioloides magna* ES.

Differential staining of purified 26-28 kD coproantigen on SDS-PAGE, under reducing and non-reducing conditions, indicated that the coproantigen was a monomeric, highly glycosylated glycoprotein. Alkaline treatment of the purified coproantigen resulted in an 8 KD protein core which still contains the epitope recognized by the MAbs. No protease activity was associated with the 26-28 kD coproantigen. The coproantigen was cleaved by trypsin without altering the reactive epitope, but was resistant to pepsin treatment. The coproantigen was stable under several different storage conditions. Indirect immunofluorescence on tissue sections of adult flukes indicated that coproantigen was present on gut cells and tegument.

A capture enzyme-linked immunosorbent assay (ELISA) was developed for the detection of the 26-28 kD coproantigen. Monoclonal antibody M2 DS/DSF10 was used

to capture the antigen from the feces and hyper-immune rabbit ant-26-28 kD glycoprotein followed by peroxidase labeled goat anti-rabbit immunoglobulin was used for detection. The assay was able to detect down to 300 pg coproantigen /ml. Feces from 27 experimentally-infected calves with known numbers of flukes were used to evaluate the test. Pre-infection feces from the same animals were used as negative controls. Coproantigen was detected in feces of calves with more than 10 flukes.

In conclusion, both the coproantigen capture ELISA and WB were both more sensitive than microscopic examination for the diagnosis of *Fasciola* infection and both could detect pre-patent infections. Moreover, the ELISA for the detection of coproantigen was easier to perform.

CHAPTER I

INTRODUCTION

RATIONAL FOR THE PRESENT STUDY:

Fasciola hepatica is a cause of major economic losses to the cow-calf, stocker and feedlot phases of the United States cattle industry. Moderate to heavy *F. hepatica* infections (over 40 flukes) result in production losses in cow-calf operations in the major enzootic areas of the Southeastern and Northwestern US and in stocker operations in the Midwest and elsewhere that receive calves from fluke zones. If the flukes are not removed by treatment or partial self-cure, performance losses are passed on to feedlot operators, along with liver condemnation losses.

Fasciola hepatica occurs in the bile ducts of ruminants, other animals and man. Infection with *Fasciola* occurs by ingestion of the encysted metacercaria on the grass. Following excystation in the duodenum of the host, the immature flukes penetrate the intestine, wander in the abdominal cavity for 4-6 days and then penetrate the liver capsule. Immature flukes migrate in the liver parenchyma for 5-6 weeks, then enter the bile duct where they mature and start egg shedding approximately 8 weeks after infection. The eggs enter the intestine with the bile and leave the host in the feces. In appropriate enviromental conditions eggs develop on pasture in water and hatch in about 10-12 days producing the miracidia. Further development requires a snail of the Lymnaeid group. The miracidium actively penetrates the snail where it develops in the hepatopancreas sequentially into

sporocyst, rediae and cercariae. The cercariae leave the snail 5 to 7 weeks after infection. Cercariae are attracted to light and green color characterestic of the plants at the margin of habitats. Cercariae adhere to the vegetation, lose their tails and form a cyst wall that protect cercaria until ingestion by the final host.

Definitive diagnosis of fasciolosis is usually achieved by traditional parasitologic methods that depend on identifying eggs in the feces. However, *F. hepatica* does not mature and shed eggs until two month after infection, and in many instances significant damage has already occurred in the host before a clear diagnosis is possible. Also *F. hepatica* eggs may be confused with eggs from other trematode parasites such as *Cotylophoron cotylophorum* and *Paramphistomum* spp. Moreover standard fecal sedimentation procedures for quantitating fecal egg counts are time consuming (15-30 min per sample) and prone to technical error. Examinations must be done on at least 10 animals per herd or purchase lot due to parasite overdispersion, the normal biological variation of infection intensity that occurs between individuals in a population, and because stocker-feedlot calves are typically grouped from several sources that may or may not be from fluke areas.

An immunodiagnostic test may overcome many of these difficulties and provide more accurate, sensitive and rapid means for the diagnosis for fasciolosis. While serodiagnostic tests rely on the detection of antibodies in the infected host, these tests only indicate exposure to the parasite and may not be related to current infection burdens, especially after treatment. Tests based on detection of circulating parasite antigens indicate

active infection; however, these circulating antigens tends to form immunocomplexes resulting in lower sensitivity of the assay. Moreover, these antigens may not be available in the circulation after the fluke is established in the bile duct. Detection of parasite-specific antigens in the feces has been successfully used for diagnosis of several parasites. Coproantigen diagnosis has the advantage of detecting only active infections. In addition, coproantigen should be detectable as long as the fluke is present in the bile duct and therefore can be used to detect both acute and chronic infection. The goal of this work was to develop a sensitive, rapid diagnostic test to detect coproantigen in the feces of infected cattle.

OBJECTIVES:

The overall objective of this study was to develop a rapid, sensitive and specific procedure for detection of mature and pre-patent *F. hepatica* infection by detection of coproantigen in infected cattle. In previous work in our laboratory, a 26-28 kD diagnostic coproantigen was detected by WB using bovine and rabbit serum and four monoclonal antibodies had been developed against this band. The specific objectives of the current work are:

1. To confirm the specificity of the 4 Mabs for the 26-28 kD coproantigen present in the feces of cattle infected with *F. hepatica* using WB and to

determine the sensitivity of the Mabs based WB for diagnosis of *F. hepatica* infection.

2. To identify the biochemical nature of the 26-28 kD coproantigen and determine the stability of the antigen under different conditions.
3. To determine the location of the 26-28 kD coproantigen on the bile duct stages of *F. hepatica.*
4. To develop an ELISA for diagnosis of *F. hepatica* using MAb and polyclonal antibodies specific for the 26-28 kD coproantigen in the feces of cattle

LITERATURE REVIEW:

Definitive diagnosis of fasciolosis in is usually achieved by traditional parasitologic methods that depend on identification of eggs in the feces. These procedures are time consuming and inefficient indicators of infections since egg counts are variable, and in cattle are commonly less than 5 eggs per gram of feces, even in heavily infected herds. Moreover, herd egg counts peak and wane depending on seasonal transmission (Malone and Craig, 1990). Duwel and Reisenleiter (1990) monitored egg shedding in bulls experimentally infected with different numbers of *F. hepatica* metacercaria *Fasciola* eggs fluctuated within individual animals and within one infection group, and the distribution of *Fasciola* eggs in the feces was irregular within a single day and over several days in all animals. *F. hepatica* matures and lays eggs approximately 8 weeks after infection. Acute symptoms occur early in the infection, due to the migration of the fluke in the liver;

therefore, eggs are not in the feces during acute fasciolosis. Moreover, in many cases, *F. hepatica* eggs may be confused with eggs of other trematode parasites such as *Cotylophoron* sp. and *Paramphistomum* spp. An immunodiagnostic test may overcome many of these difficulties in the diagnosis of fasciolosis and provide more accurate, sensitive and rapid means of diagnosis.

Serological diagnosis:

As early as 1950, Oliver-Gonzalez et al., developed an intradermal test to differentiate between *F. hepatica* infected and non-infected cattle. This test and similar tests (Maekawa et al., 1954; Maekawa and Kushibi, 1965; Pautrizel et al., 1962) demonstrated cross-reactivity in cattle with tuberculosis and in patients with schistosomiasis (Rifaat and Abdel-Aal, 1968).

Early approaches in the serodiagnosis of fasciolosis included complement fixation test (CF) and indirect fluorescence assay (IFA). The CF test proved to be unreliable (Platzer, 1970). IFA using *F. hepatica* miracidia was used to diagnose infections in sheep and man (Faraga de Acevedo and Rombert, 1965). This IFA had a sensitivity of 74-80%, but some cross-reactivity was seen with the sera of human patients with *Schistosoma haematobium.* Other reports used sections from the adult flukes in IFA for diagnosis of *F. hepatica* (Coudert et al., 1967; Deelder, 1973). Hanna and Jura (1977) used freshly excysted *F.gigantica* as an antigen for an IFA test to monitor experimentally infected animals. Antibodies were detected 2-6 weeks post-infection and diminished gradually to

the pre-infection levels by 22 weeks post-infection, which suggested there were difference in antigenicity of juvenile and mature flukes.

To develop more specific immunological tests for diagnosis of *F.hepatica,* a number of authors intiated investigations directed at increasing knowledge on the detected antigens. Numerous antigens have been identified in the tegument of *F. hepatica.* Hillyer (1980) isolated *F. hepatica* tegument antigens and used them for serodiagnosis of experimentally infected rabbits using immunodiffusion against the rabbit serum. He also found that the tegument contained antigens that protected mice from challenge infection with *Schistosoma mansoni.*

The tegument of the *F. hepatica* consists of two main layers; the outer zone, or the distal cytoplasm, where the spines are embedded, and the proximal cytoplasm which contain the tegumental cells. The distal cytoplasm is coated by the glycocalyx which is sloughed continuously. The proximal cytoplasm cells are responsible for maintaining the glycocalyx. Proximal and distal cytoplasm are connected by tubules underlying these tegumental cells and the translocation of the content of these cells to the distal cytoplasm takes place through these connecting tubules (Bennett and Threadgold, 1973, Bennett and Threadgold, 1975). Hanna (1980a) reported that in the newly excysted juvenile these tegumental cells are unique and he designated them as T0 cells. During migration to the liver the T0 cells metamorphose into T1 cells which, although morphologically distinct, are produced in the same perikarya and may possess similar antigenicity and function. The T1 cells secret T1 granules. Upon entering the bile duct as immature flukes, T2 cells, which

arise from different perikarya, start to appear; and for a transitional period the glycocalyx is formed by both the T1 and T2 granules, followed by a decrease in the number of T1 cells (Hanna 1980a). Hanna and Trudgett (1983) raised 6 MAbs against the tegumental antigen present in T1 granules and the glycocalyx of the flukes. These MAbs were found to bind the same T1 epitope. They found that T1 type antigen consisted of a polypeptide with a MW of 50 kD, possibly linked to smaller entities with a MW of 25- 40kD.

Santiago de Weil et al. (1984) isolated *F. hepatica* antigens which induce antibody formation in acute fasciolosis. These antigens, when fractionated through Sephacryl S-200 column, resulted in three major peaks. The best serologic antigens were found in peak 2 (MW of 14 kD-43 kD). This peak contained *F. hepatica* genus specific antigens that did not cross-react with either *Schistosoma mansoni* antigens or with bovine serum albumin. By WB, using rabbit antiserum raised against peak 2, Santiago de Weil et al. (1986a) found that the antigen consisted of one main polypeptide of MW of 12 kD, and 6 additional peptides in the 26-62 kD range.

Santiago and Hillyer (1986b) identified a 23 - 28 kD polypeptide complex of *F. hepatica* ES, which induced an antibody response in both acute and chronic fasciolosis. Hillyer and Soler de Galanes (1988) identified an *F.hepatica* antigen of 17 kD which was recognized by the serum of humans, calves, sheep and rabbits with fasciolosis, and did not cross-react with sera from individuals infected with *Schistosoma mansoni* or *Trichinella spiralis*. They suggested that the 17 kD antigen might be an excellent candidate for immunodiagnosis of acute and chronic fasciolosis.

Enzyme linked immunosorbent assay (ELISA) has been used by a number of investigators in efforts to develop tests for serodiagnosis of fasciolosis. Burden and Hammet (1978) used the ELISA to detect bovine fasciolosis using crude *F. hepatica* worm extract. Hillyer (1978) adapted the ELISA for serodiagnosis of *F. hepatica* in rats using partially purified crude antigen, and reported that the antibody titer rose by the second week of infection in rats, reached its highest level 4 weeks post-infection and remained high through 12 weeks post-infection. In a similar study in rabbits, Hillyer and Santiago de Wiel (1979) found that the antibody titer was the highest by the fourth week post-infection and remained high through 28 weeks post-infection. Farrell et al. (1981) detected antibodies to *F. hepatica* in cattle as early as 4 weeks post-infection using freshly collected fluke extracts. Zimmerman et al. (1982), used an ELISA and fresh fluke antigen to detect antibodies to bovine *Fasciola* infection as early as 2 weeks after infection. Oldham (1983) detected anti *F. hepatica* antibodies 3 weeks after infection using a semi-purified high MW antigen. Hillyer et al. (1985) monitored *F. hepatica* experimentally infected calves by gel diffusion, counterelectrophoresis (CEP) and ELISA using crude antigen; serological diagnosis was positive as early as 2-4 weeks of infection by gel diffusion, by 4 weeks by CEP, and by 6-8 weeks by ELISA, whereas parasitologic diagnosis via the detection of flukes eggs in the feces was possible only after 8-10 weeks. Zimmerman et al. (1985) developed a test for ovine fasciolosis using dot ELISA that successfully detected the antibodies 4 weeks after infection. Wyckoff and Bradley (1986) developed an ELISA for quantitative diagnosis of bovine fasciolosis. They detected the antibodies as early as 2

weeks post-infection and also found a correlation between the antibody level and the fluke burden. Similar results were obtained for sheep and cattle by Santiago and Hillyer (1988). Using ELISA, Espino et al. (1987) used ES of *F. hepatica* to detect anti-*F. hepatica* antibodies in sera from human patients with fasciolosis without cross-reactivity in serum from patients with schistosomiasis, clonorchiasis, ascaris or hookworms. Carlos et al. (1988) evaluated an immunodiagnostic antigen in the ES product of *F. hepatica* after fractionation by gel filtration, and found that a component with a MW of 150-160 kD was very reactive with sera of rabbits with early fasciolosis. They also found that serum from both acute and chronic fasciolosis recognized a prominent 25-30 kD polypeptides component. These polypeptides were also recognized by sera from infected sheep and cattle. Fagbemi and Guobadia (1995) used a 28 kD cysteine protease of *F. gigantica* adult worm as antigen for immunodiagnosis of fasciolosis in cattle, sheep and goats. Several other ELISA, dot ELISA and FAST ELISA tests have been developed by modifications of the above tests and used for diagnosis of fasciolosis in human and animals (Sinclair and Wassall (1988); Shaheen et al. (1989); Hillyer and Soler de Galanes (1991); Hillyer et al. (1992); Yang and Lan (1992); Richard (1995).

Another serological method that has been used successfully for early diagnosis of fasciolosis is the detection of specific anti-f2 antigen using hemagglutination tests. F2 antigen has a tegumental origin and has been shown to be specific and immunogenic (Hillyer, 1980; Santiago de Weil et al., 1984). Levieux et al. (1992a) used this assay to detect anti-*Fasciola* antibodies in experimental infected calves as early as 2-4 weeks post-

infection and the titer persisted at high levels during the 28 week experimental period. This test was evaluated with the respect to its potential use in the diagnosis of caprine fasciolosis by Levieux et al. (1994b) who detected the f2 specific antibodies at 2-3 weeks post-infection, with the maximum titer at 9 weeks after infection, after which the antibody titer declined.

Circulating antigen:

Although the use of ELISA for detection of antibodies in the infected host is a valuable diagnostic method, especially in detection of early infection, the persistence of antibodies for a long period after treatment makes differentiation between the exposure to the parasite and active infection difficult (Hillyer,1993). Recent studies have focused on the immunodiagnosis of *F. hepatica* by detection of *Fasciola* circulating antigens. Antigen detection has the advantage over antibody-based tests in that the antigenemia implies current infection. Sampaio- Silva et al. (1981) detected immunocomplexes in sera of *Fasciola* infected patients using a CIq binding assay and found that only 36% of the previously confirmed positive samples were positive by the test. Using a two-site ELISA Langley and Hillyer (1989a) detected circulating parasite antigen in murine fasciolosis as early as 1 week post-infection with the maximum detection level at 3 weeks after infection. Langley and Hillyer (1989b) detected circulating immunocomplexes in sera of 5 *F. hepatica* infected calves with fluke burdens ranging from 27 to 70 flukes. Circulating immunocomplexes were detected 6-8 weeks after infection, returned to approximately pre-infection levels and then rose slightly thereafter. With capture ELISA, utilizing a

monoclonal antibody to capture the circulating *F. hepatica* antigens in human sera, and peroxidase conjugated human polyclonal antibody to identify these circulating antigens, Espino et al. (1987) detected antigen concentration as low as 10 ng/ml; there was no cross-reactivity with other parasite antigens. Rodrigue-Pérez and Hillyer (1995) used rabbit anti-*F. hepatica* ES and sheep anti-*F. hepatica* ES in a capture ELISA to detect circulating antigen in experimentally infected sheep. They were able to detect antigen as early as 4-6 week post-infection with maximal detection at 8 weeks post-infection.

Coproantigen:

While serum antigen detection methods have advantages over serum antibodies detection methodes, the use of serum samples to detect parasite antigen has two disadvantages: first, circulating antigen tends to form immunocomplexes with host antibody which deceases the potential rate of detection (Langley and Hillyer, 1989b) and Second, The collection of serum samples require animal handling and are often inconvenient to obtain, especially for herd evaluations. Diagnosis of parasitic infection by detection of parasite specific antigen in the feces was first reported in 1962, by Bobos and Nemith, who used hyperimmune rabbit serum raised against hydatid cyst fluid to detect *Echinococcus granulosus* coproantigen using the double gel diffusion. Antigen was present before the infection became patent, but the test cross-reacted with samples from taenia-infected individuals.

Among protozoan parasites, Craft and Nelson (1982), detected *Giardia* antigen in human feces by counterimmunoelectrophoresis. ELISA was later used to detect coproantigen in human *Giardia* infections. Using anti-trophozoite hyperimmune goat serum to capture the antigen from feces and anti-trophozoite hyperimmune rabbit serum to detect the bound antigen, Ungar et al. (1984) developed an ELISA for detection of *Giardia lamblia* in human feces. The assay detected the antigen in 92% of patients with giardiasis. Patients who received treatment became negative by the test. The usefulness of ELISA for detection of the antigen in the feces was determined in experimentally infected humans by Nash et al. (1987) who concluded that the test is at least as sensitive as microscopic examination for diagnosis of *Giardia* infections and that the ELISA is easier to perform. In a parallel study, hyperimmune rabbit anti-*G. lamblia* cyst serum was used to detect *G. lamblia* coproantigen (Rosoff and Stibbs, 1986) by CEP and WB. A diagnostic *G. lamblia* coproantigen was identified that had a MW of 65 kD (GSA 65). The antigen was specific for *G. lamblia*. Stibbs et al. (1988) developed a capture ELISA test for detection of GSA 65 antigen in the feces of infected patients. They found the test more sensitive than the trophozoite- derived antigen assay. Rosoff et al.(1989) evaluated a commercially available ELISA for detection of GSA 65 in a clinical trial and found that the ELISA performed well on fecal samples treated with 10% natural formalin, sodium acetate-formalin fixative, and Cary-Blair transport medium. Vinayak et al. (1991) developed a dot ELISA for detection of *G.lamblia* antigen in stool eluates using clinical cases of giardiasis. They concluded that their test was easy, rapid, sensitive and specific

and that it could detect the subclinical cases that were negative by microscopic examination. Ungar (1990) developed a capture ELISA for detection of *Cryptosporidium* antigens in human fecal

specimens using specifically produced goat and rabbit anti-*Cryptosporidium* antigens. *Cryptosporidium* detection ELISA kits are now commercially available and have been evaluated (Siddon et al., 1992; Newman et al., 1993). El-Shewy et al. (1994) identified *Cryptosporidium parvum* coproantigens of 18 and 20 kD in stool eluates of calves and humans infected with *Cryptosporidium* species. Both of these bands were recognized by monospecific antibodies raised against the 20 kD antigen which reacted with *C. parvum* trophozoites by IFA. The antigens remained detectable in fecal samples stored with commonly used preservatives and at various temperatures. For amoebiasis, several assays involving both monoclonal and polyclonal antibodies had been developed (Ungar et al., 1985; Jain et al., 1990; Agarwal et al., 1991; Vinayak et al.,1993). In 1994, Haque et al., developed a capture ELISA for detection of pathogenic *Entamoeba histolytica* coproantigen using purified rabbit polyclonal antibody against the galactose adhesion molecule to trap the antigen, and a MAb directed against a pathogen-specific epitope of that molecule for detection. They found their test had a specificity of 97% and sensitivity of 100%. Sharma et al. (1994) developed an ELISA to distinguish the pathogenic *E. histolytica* from the non-pathogenic *E. dispare* using MAbs directed against cross-reactive and *E. histolytica*-specific epitopes of galactose adhesion molecule. Gonzalez-Ruiz et al. (1994) used an

invasive *E. histolytica* strain-specific monoclonal antibody in a capture ELISA and detected the fecal antigen with a sensitivity of 87% and specificity of 100%

Coproantigen detection tests have been developed for diagnosis of several cestodes. Allan et al. (1989) developed a capture ELISA for detection of *Hymenolepis diminuta* coproantigen in fecal supernatant of infected rats. They found that fecal antigen levels could be detected one week after infection with the maximum antigen detectable at 3 weeks. Following drug treatment of the infected rats, there was an initial steep rise in fecal antigen levels, followed by decline to the pre-infection level. Deplazes et al. (1990) used affinity-purified polyclonal antibodies raised against ES antigens of adult *Taenia hydatigena* to detect antigen in fecal samples of infected dogs. The antigen was first detected at 18 days after infection and showed no cross-reactivity with other cestodes or nematodes. Allan, et al. (1990) developed a capture ELISA for detection of coproantigen from *Taenia solium* and *Taenia saginata* infected hosts; although there was no cross-reactivity between the two species when fecal samples from experimentally infected hamsters were used, cross-reactivity was detected when human fecal samples were used. Patients previously diagnosed as positive became negative 6 days post-treatment. Deplazes et al. (1990) used affinity-purified polyclonal antibodies raised against ES antigens of adult *T. saginata* to detect coproantigen and found the antigen in 85% of the fecal sample of infected patients, whereas eggs were detected in only 62% of the cases. Maass et al. (1992) isolated the immunodiagnostic coproantigen from fecal samples of known *T. solium* carriers by several chromatographic purification methods. The 60 kD protein, a major

antigenic component excreted with the feces, was shown to react by WB with polyclonal anti-*T. solium* IgG. Allan et al. (1993) used antibodies raised against adult *T. solium* in immunosuppressed, experimentally infected hamsters to develop a dipstick dot ELISA for the detection of *Taenia* coproantigen in people. The test showed 99% specificity. Some of these tests were applied in field studies carried out in China and Guatemala that revealed a high specificity (Allan et al.1992). In the same study, antiserum against *T. pisiformis* antigen extract was used to detect the coproantigen in *T. pisiforms* of experimentally infected dogs. They found that the antigen was present in the feces before patency and antigen levels were independent of *T. pisiformis* egg output.

Following the pioneering work of Babos and Nemeth (1962), coproantigen of *E. granulosus* was later detected by ELISA in *E. granulosus* infected dogs using rabbit hyperimmune serum against saline extracts of the non-gravid proglottids of *E. granulosus* (Allan et al., 1992). The test had 87.5% sensitivity and 95% specificity when tested against samples from dogs naturally infected with *Echinococcus* or *Taenia*. Walters and Craig (1992) used this test in the field and found it to be convenient and highly sensitive. Using protein-A-purified polyclonal antibodies against ES of *Echinococcus*, Deplazes et al. (1992) developed an ELISA for detection of *Echinococcus* coproantigen in fecal samples of dogs, dingoes and foxes infected with either *E. granulosus* or *E. multilocularis* . They were able to detect *E. granulosus* antigen 10-20 days post-infection and *E. multilocularis* antigen 5 days post-infection, but the test was not sensitive enough for detection of low level infections (< 70 worms). Baronet et al. (1994) used the ELISA protocol described

by Allan et al. (1992) to detect *E. granulosus* coproantigen in a field study using rabbit hyperimmune serum specific for adult *E. granulosus* (proglottids) somatic antigen. The test had a sensitivity of 88% for burden of >15 worms. Two ELISA immunodiagnostic tests based on genus-specific coproantigen detection or serum antibody detection were compared with arecoline purgation for detection of *Echinococcus* in naturally infected dogs in Uruguay (Craig et al., 1995). The coproantigen ELISA was more sensitive than the ELISA for anti- *Echinococcus* serum IgG. Moreover, coproantigen reactivity was positively correlated to purge worm count, but there was no correlation of antibody levels with worm count.

For coproantigen diagnosis of the intestinal nematodes, Ellis et al. (1993) developed an antigen capture ELISA that could identify *Haemonchus contortus* antigen in feces from naturally infected sheep with moderate parasite burdens. The ELISA used rabbit or sheep polyclonal antibody to capture the coproantigen. The captured antigen was detected by a mouse MAb specific for a cuticle surface antigen of *H. contortus*. Characterization of the reacting antigen using the MAb indicated that the antigen was a collagenase and protease resistant surface protein of *H. contortus* with a MW of 122, 65, and 49 kD on SDS-PAGE under reducing conditions. Nageswaran et al. (1994) investigated the potential for coproantigen detection for diagnosis of strongyloidiasis in rats experimentally infected with *Strongyloides ratti*. A capture ELISA was developed using affinity-purified polyclonal antibodies raised in rabbits against adult and filariform larvae somatic antigens. Using anti-larval antibodies they detected the coproantigen 5 days after infection and coincided with

the appearance of the eggs and larvae in the feces. Peak antigen level was detected 9 days after infection and remained relatively high until day 25 post-infection. The same results were obtained when anti-adult somatic antigen capture ELISA was used, but coproantigen was detectable until day 46, when the experiment was terminated.

The first report of coproantigen diagnosis of flukes was for *Opisthorchis felineus* (Teplukhin et al., 1986). Using passive hemagglutination and antibody neutralization they detected the antigen in the feces of *Opisthorchis*-infected human patients. Sirisinha et al. (1991) later reported a potentially useful MAb-based ELISA for the detection of coproantigen from a small number of patients with opisthorchosis. The method was based on the detection of a 89 kD *O. viverrini* metabolic antigen present in the feces. The assay was estimated to be sensitive enough to detect antigen produced by a single mature fluke. Sirisinha et al. (1995) later evaluated this MAb-ELISA based assay for the diagnosis of *O. viverrini* infection and showed that the ELISA was sufficiently sensitive and specific for the diagnosis of *O.viverrini.*

Youssef et al. (1991) reported a counterimmunoelectrophoresis method that used hyperimmune serum from rabbits immunized with partially purified *Fasciola gigantica* antigen to detect coproantigen in saline extract of human patients' stools. The test was specific and detected the antigen 3-4 weeks before microscopic detection of the eggs. El-Bahi et al. (1992) identified a 26 kD diagnostic *F. hepatica* antigen in the bile, intestinal content and feces of *F. hepatica* infected cattle by WB using hyperimmune rabbit serumraised against fresh fluke antigen . Four MAbs were developed against the 26-28 kD

coproantigen as part of their studies and these antibodies were used for the research reported in this dissertation. Espino and Finlay (1994) laterf developed a capture ELISA for detection of a fecal antigen in human fasciolosis. That test utilized a MAb to capture the antigen and rabbit anti-ES polyclonal antibody to detect the antigen. They reported that the test was specific and that the antigen was not found in the feces of the treated persons.

CHAPTER II

DETECTION OF A *FASCIOLA HEPATICA* 26-28 kD DIAGNOSTIC COPROANTIGEN BY WESTERN BLOT ANALYSIS USING MONOCLONAL ANTIBODIES

INTRODUCTION:

Fasciola hepatica is one of the most economically important trematode parasites of domestic animals. Definitive diagnosis of fascioliasis is usually achieved by the identification of eggs in the feces; However, *F. hepatica* does not mature and shed eggs until two month after infection, and in many instances significant damage has occurred to the host before a clear diagnosis is possible (Hillyer, 1986). Serodiagnostic tests for the detection of antibodies in infected animals have been developed (Hillyer et al., 1985; Zimmerman el al., 1985; Welch et al., 1987; and Hillyer and Soler de Galanes, 1991) These tests can identify animals that are infected before mature worms are present, but antibody levels may not be related to current infection burdens, especially after treatment. Detection of circulating parasite antigen indicates active infection, however, the collection of serum samples require animal handling which are often inconvenient to obtain, especially for herd evaluations. Diagnosis of parasitic infection by detection of parasite specific antigen in the feces was first reported in 1962, by Bobos and Hemeth, who used hyperimmune serum to detect *Echinococcus granulosus* coproantigen using the double diffusion technique. In 1982, Craft and Nelson detected *Giardia* antigen in human feces

by counter immunoelectrophoreses. ELISA was later used to detect coproantigen in human *Giardia* infection (Ungar et al., 1984; Green et al.1985; Rosoff and Stibbs, 1986), and *Cryptosporidium* infection (Ungar, 1990) and ELISA diagnostic tests for both parasites are now commercially available. ELISA methods have also been developed for detection of coproantigen of cestodes. Using hyperimmune sera, coproantigen has been detected in *Echinococcus granulosus* infected dogs (Allan et al., 1992; Deplazes et al., 1992, Deplazes et al., 1994; Walters and Craig, 1992; Craig et al., 1995.), *Taenia pisiformis* infected dogs (Allan et al.,1992), *Hymenolepis diminuta* infected rats (Allan et al., 1989), as well as *Taenia solium* and *Taenia saginata* infected human (Allan, et al., 1990; Mass et al., 1992; Allan et al.,1993). More recently intestinal nematodes have been identified using immunodetection of coproantigen. Coproantigen of *Strongyloides ratti* in rats (Nageswaran et al., 1994) and *Haemonchus contortus* in sheep (Ellis et al.,1993) have been detected.

Coproantigen was used for the detection of the liver trematode *Opisthorchis felineus* by Teplukhin et al. (1986) using indirect hemagglutination and by Sirisinha et al. (1991) using ELISA. *Fasciola gigantica* coproantigen was detected in human feces using counterimmunoelectrophoresis (Youssef et al., 1991). El-Bahi et al. (1992) reported detection of a 26 kD coproantigen from the bile, intestinal content and feces of *F. hepatica* infected cattle using hyperimmune bovine and rabbit serum in western blot analysis, and Espino and Finlay (1994) were able to detect the coproantigen in human fasciolosis using sandwich ELISA.

Current procedures for diagnosis of *Fasciola* infection by fecal egg counts are time consuming and prone to technical error, especially for herd evaluations (Malone and Zukowski, 1992). Moreover, it is not possible to detect infection before egg shedding and mature flukes are present. The objective of the current study was to develop a sensitive and specific procedure for detection of mature and pre-patent infections in *F. hepatica* infected cattle. I describe here detection of coproantigen in cattle using monoclonal antibodies (MAbs) in a WB and development of a test for detection of low worm numbers and the premature infection by detection of coproantigen in cattle. This coproantigen-based test offers several advantages over current serologic tests: 1) fecal samples can be collected without animal handling; 2) the same test can be used for several host species; 3) only current infections are detected and 4) quantitation of fluke burden may be possible.

MATERIAL AND METHODS:

Monoclonal antibodies:

Six to eight weeks old female BALB/c were immunized intraperitonially (IP) with 20-52 μg/ml *F.hepatica* ES and boosted, IP, with the 26-28 kD protein blotted on a nitrocellulose membrane from a gel loaded with an equivalent amount of *F hepatica* (Harlow and Lane, 1988). Fusion of the mouse myeloma cell-line SP2/O (American Type Culture Collection, ATCC, Bethesda, MD) with spleen cells of the immunized mice was performed as previously described (St. Groth and Scheidegger, 1980). Hybridoma culture supernates were screened for the presence of antibodies against ES and fresh fluke antigen

(FFA) using ELISA according to the method of Santiago and Hillyer (1988). Four hybridomas producing antibodies, M2DS/DSF10 (F10), M7 D5/G7 (G7), M1CS/E7 (E7) and M7D5/A11F7 (F7) were identified and cloned by limiting dilution. Ascites fluid was produced from each hybridoma according to the method of Gallate (1987). These MAbs were determined to be of the IgM/κ isotype using the SEA Fisher Biotech Clonotyping System II (Fisher Scientific, Pittsburgh, PA). Monoclonal antibodies were purified from ascites using ImmunoPure IgM Purification Kit (Pierce Chemical Co, Rockford, IL). Protein concentrations were determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories Inc., Hercules, CA) according to the manufacturer's instruction. Antibody concentrations were adjusted to 1:1.4 mg/ml protein and stored at - 20°C. MAbs were biotinylated using ImmunoPure NHS-LC- Biotin (Pierce Chemical Co, Rockford, IL) according to the manufacturer's instructions and stored at 4°C.

Animals:

Yearling cattle were obtained from a fluke free area and were confirmed to be fluke-free by using a sieve-sedimentation egg count procedure (Flukefinder, Mosco, ID). Each animal was experimentally infected with 500 metacercarae. Fecal samples were collected 6, 8, 11, 13 and 14 weeks post-infection for fecal egg counts. Sedimentation and egg count were performed as above except that 6 gm of feces were used instead of 2 gm to increase the sensitivity. Fecal samples were stored at 4 °C. Animals were slaughtered 14 weeks after infection and the number of flukes recovered from each animal was determined at necropsy by the method of Malone et al. (1982).

Excretory secretory product (ES):

F. hepatica ES was prepared as described by Santiago and Hillyer (1986). Briefly, adult flukes were removed from the bile duct of naturally infected cattle and washed 3 to 4 times in a 1 hr period at room temperature with 0.01 M phosphate buffered saline, pH 7.2 (PBS) in order to remove all traces of blood and bile and to allow flukes to regurgitate cecal contents. Flukes were then incubated in PBS, at 37°C for 3 hr. After incubation, the solution was collected and centrifuged at 5,000 x *g*. The supernatant was collected and concentrated to 1/5 its original volume using Centriprep 10 concentrators (Amicon Inc., Beverly, MA). Protein concentration was determined using Bio-Rad Protein Assay according to the manufacturer's instructions and the ES was stored at - 20°C .

Fresh fluke antigen (FFA):

Fresh fluke antigen was prepared by the method of Farrell et al. (1981). After washing flukes for 3 min in PBS, 1 ml of PBS per gram of parasite was added and flukes were ground in a mortar until no visible tissue particles remained. Fluke tissue was then homogenized using a Tenbroek tissue grinder to obtain a uniform suspension and stored over night at 4°C. The homogenate was then centrifuged at 12,000 x g for 30 min. Supernatant was collected and its protein content was determined using Bio-Rad Protein Assay. The FFA antigen was stored at - 20°C. Excretory secretory product and FFA of *Paramphistomum microbothriodes* and *Moniezia sp.* were prepared by the same method. *Fascioloides magna* ES and FFA were provided by Dr. B.E. Stromberg, University of Minnesota St Paul, MN.

Fecal sample preparation:

Fecal supernatant were prepared for coproantigen immunodetection according to El-Bahi et al. (1992). Briefly, 5 gm of feces were mixed with an equal amount of distilled water and sonicated for 5 min (150 Watt interrupted pulse output at 50% duty cycle using a Sonifier Cell Disrupter Model W 350). The fecal suspension were centrifuged at 1900 x g for 15 min. The supernatant was dialyzed in 6-8 kD dialysis tubes overnight at 4 °C against 2 changes of PBS. Fecal supernatant were then concentrated to 1/5 of the original volume by absorption against polyvinyl pylorridone 36 kD and stored at -20°C until use.

Sodium dodocyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting:

Sodium dodocyl sulfate polyacrylamide gel electrophoresis was performed by the method of Laemmli (1970). A 4% stacking gel and a 12% separating gel were used. Samples were diluted 1:2 in 0.625 M Tris-hydrochloride pH 8.0, 10% (v/v) glycerol , 2.3% (w/v) SDS and 5% (v/v) 2 - mercaptoethanol (sample buffer) and boiled at 100°C for 5 min. Ten µl of the diluted samples in the sample buffer were applied to SDS-PAGE gel at 150 V. Proteins were transferred from polyacrylamide gel to immobilon-P transfer membrane (Millipore Corporation, Bedford, MA) and the blotted protein were analyzed by the method of Towbin et al.(1979) . Briefly, membranes were soaked overnight in blocking buffer consisting of 0.05M Tris, 0.001M EDTA and 0.15 NaCl pH 7.4 (NET) containing10 % non fat dry milk for the indirect assay or 3% fish gelatin in case of biotinylated MAbs. Membranes were washed 4 times for 10 min using NET buffer

containing 0.05% Tween 20, then transferred to 10μg/ml ofMAbsor 1μg biotinylated MAbs in freshly prepared blocking buffer. After a 1 hr incubation with gentle shaking, the membrane was washed as before and transferred to a 1:500 dilution of peroxidase-conjugated goat anti-mouse IgG (H&L) (Jackson ImmunoResearch Laboratories, Inc.. West Grove PA.) in the blocking buffer for MAbs or to a 1:10,000 dilution peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA.) in blocking buffer for biotinylated MAbs. After incubation with gentle agitation for 1 hr, membranes were washed, and substrates were added. Two substrates were used: 3.3',5,5' tetra methylebenzidine (TMB; Kirkegaard and Perry Laboratories, Gaithersburg, MD) and the Enhanced Chemiluminescence substrate (ECL; Amersham Life Science Inc., Arlington Heights, IL). Molecular weight estimates were made by comparing the mobility of the tested samples with that of the standard protein mixture(Bio-Rad Laboratories Inc.) separated on the same gel.

RESULTS:

Detection of coproantigen:

The presence of coproantigen in the feces of experimentally infected calves was determined by WB. Since the four MAbs react identically with the 26- 28 kD band of the ES, ES was used as a positive control. Known negative fecal samples were used as a negative control. By WB using culture supernatants containing MAbs and TMB, the colorometric substrate, a 26-28 kD band was detected in the ES, but the assay was not

sufficiently sensitive to detect the band in the fecal samples (Fig. 2.1). However, the 26-28 kD band was detected using the culture supernatant containing MAbs and ECL, the chemiluminesent substrate (Fig. 2.2). When MAbs purified from ascites fluid were used with the colorimetric substrate TMB, the 26-28 kD band was detected in both the ES product and in the feces from infected cattle. The sensitivity of the assay was increased further when the ECL substrate was used. To further increase the sensitivity of detection, biotin-streptavidin modification was used with both TMB and ECL substrates. Using TMB substrate, the biotin-streptavidin modification enhanced the detection of the coproantigen, however the noise level with ECL increased substantially, resulting in problems with background. I found that the indirect WB using the purified MAbs and the biotin-streptavidin WB were sufficiently sensitive; therefore, this assay system was used to determine the specificity the MAbs, and for tests on fecal supernatants from infected cattle with known number of flukes to determine the relative sensitivities of the MAbs.

Specificity of the MAbs :

F. hepatica often coexists with other trematode, nematode and cestode parasites in naturally infected cattle. To determine the specificity of the MAbs for *F. hepatica* coproantigen, WB were performed using FFA and the ES of *F. hepatica, F. gigantica, Fascioloides magna, Paramphistomum microbothioirdes* and *Moniezia* sp. All 4 Mabs reacted strongly with *F. hepatica.* The MAbs showed weak cross-reactivity with *F. gigantica* and moderate cross-reactivity with the deer fluke, *Fascioloides magna.* No

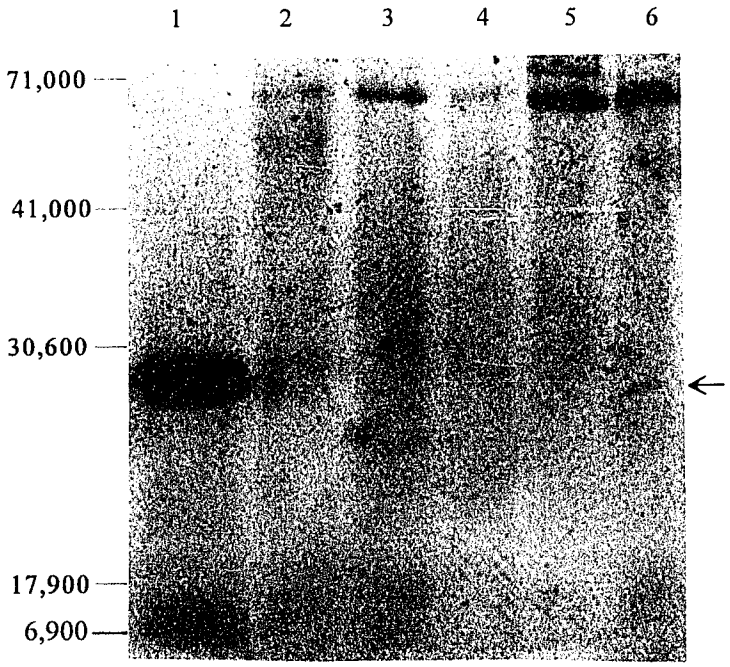

Fig. 2.1. Detection of coproantigen with MAb (F10) supernate using western blot analysis and TMB substrate; lane (1) *F. hepatica* ES product as positive control. Feces from calves infected with the following numbers of flukes; lane (2) 39, lane (3) 28, lane (4) 22, lane (5) 10 and lane (6) negative control feces.

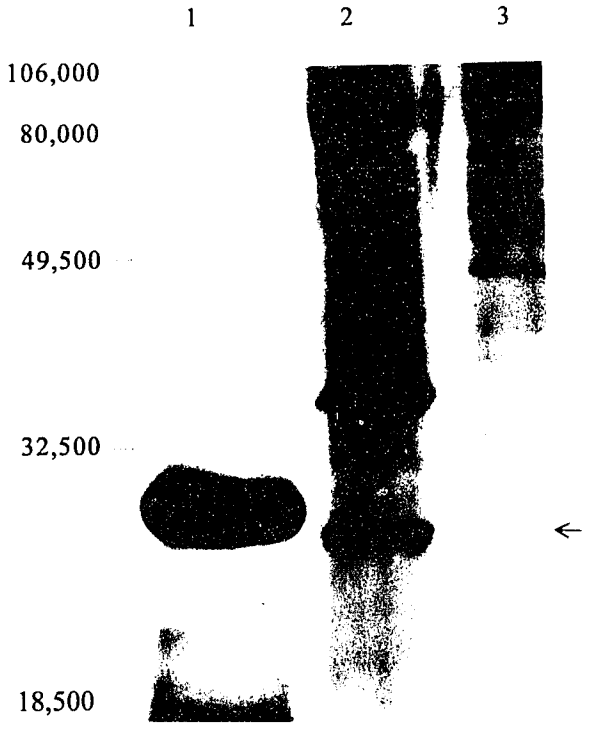

Fig. 2.2. Detection of coproantigen with MAb (F10) supernate using western blot analysis and ECL substrate; lane (1) *F. hepatica* ES product as a positive control, lane (2) feces from a calf infected with 39 flukes and lane (3) negative control feces.

reactivity was observed with the rumen fluke *P. microbothioirdes* or the cestode parasite *Moniezia sp.* (Fig. 2.3, Table 2.1).

Determination of the sensitivity of the MAbs:

The ultimate goal of this study was to develop a more sensitive procedure for detection of sub-clinical *F.hepatica* infection. To determine the sensitivity of the WB for coproantigen, feces from calves experimentally infected with 39, 28, 22, 10, 6 and 0 flukes were examined. WB were probed with either purified MAbs (indirect WB), or biotinylated MAbs (biotin-strepavidin WB). The indirect WB detected as few as 22 flukes (Fig 2.4). When the biotin-streptavidin WB were used, as few as 10 flukes were detected (Fig. 2.5). I observed that the 26-28 kD band intensity increased with increasing fluke burdens (Fig. 2.4, 2.5). The 26-28 kD band was not detected in feces from animals with fluke numbers less than 10 using either assay.

To determine whether the MAbs can detect the *F. hepatica* infection before egg laying begins by mature flukes, Streptavidin WB were performed using MAb F10 on feces from animals infected with 39, 32, 28, 22 and 10 flukes at 6, 8, and 13 week after infection. The antigen was detected in all 5 animals as early as 6 weeks post- infection. Maximum coproantigen concentration as determined by band intensity was seen at 8 weeks after infection. A representative blot is shown in Fig. 2.6. These results were compared to the number of eggs recovered by fecal sedimentation. Eggs were not recovered by fecal sedimentation from any of the calves at 6 or 8 weeks post-infection; eggs were found in 4 of 5 calves at 13 weeks post-infection (Table 2.2).

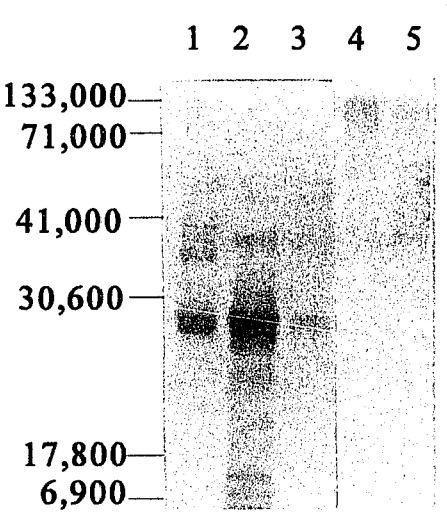

Fig. 2.3. Determination of the specificity of the Mabs for *F. hepatica* 26-28 kD antigen. Representative western blot analysis usisg MAb F 10: Lane (1) *F. magna* ES, lane (2) *F. hepatica* ES, lane (3) *F. gigantica* ES, lane (4) *Paramphistomum* sp. and lane (5) *Moneizia* sp.

Table 2.1. Determination of the cross-reactivity of the four monoclonal antibodies to common trematode and cestode parasites of cattle.

	Reactivity of the four monoclonal antibodies[a]			
	10	G7	E7	F7
F. hepatica	++++	++++	++++	++++
F. gigantica	+	+	+	++
F. Magna	+++	+++	+++	++++
P. microbothrioides	-	-	-	-
Moneizia sp.	-	-	-	-

a ++++ Very strong reactivity, +++ Strong reactivity, + Faint reaction and - no reaction

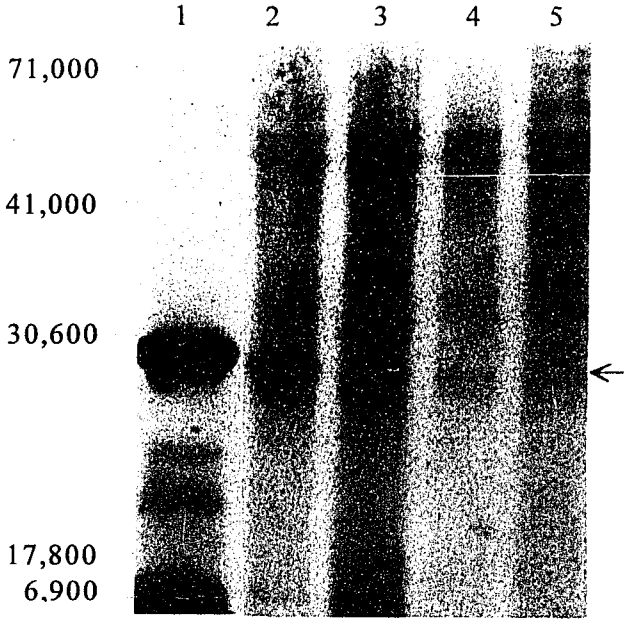

Fig. 2.4. Detection of *F. hapatica* coproantigen with purified Mab (F10) using western blot analysis and TMB as substrate; lane (1) *F. hepatica* ES product as positive control. Feces from calves infected with the following numbers of flukes: lane (2) 39, lane (3) 32, lane (4) 28 and lane (5) negative control feces.

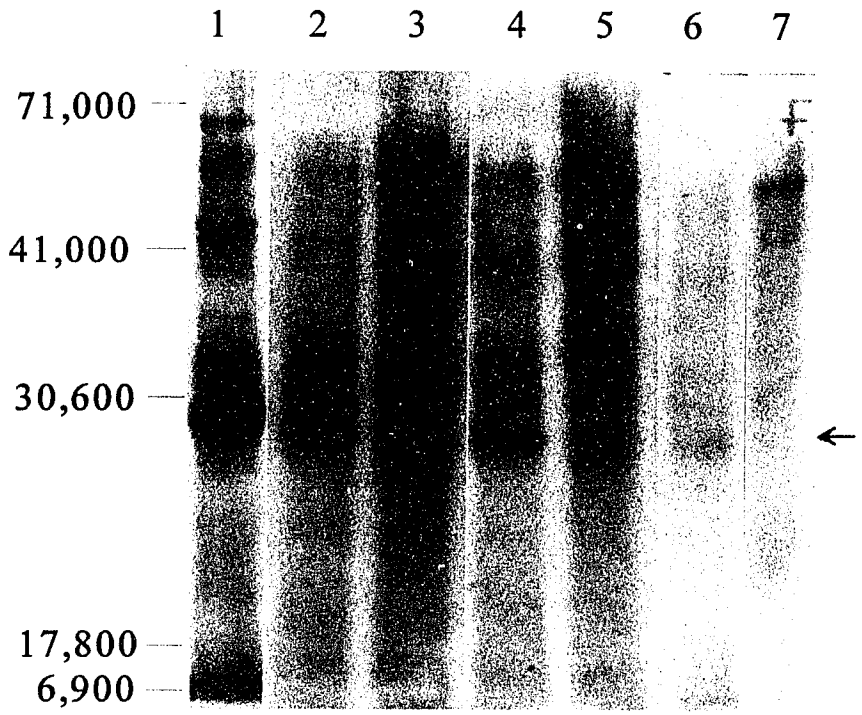

Fig. 2.5. Detection of coproantigen using a biotin-streptavidin modification of the WB and TMB substrate. Lane (1) *F. hepatica* ES product as positive control. Feces from calves infected with the following numbers of flukes: lane (2) 39, lane (3) 32, lane (4) 28, lane (5) 22, lane (6) 10 and lane (7) negative control feces.

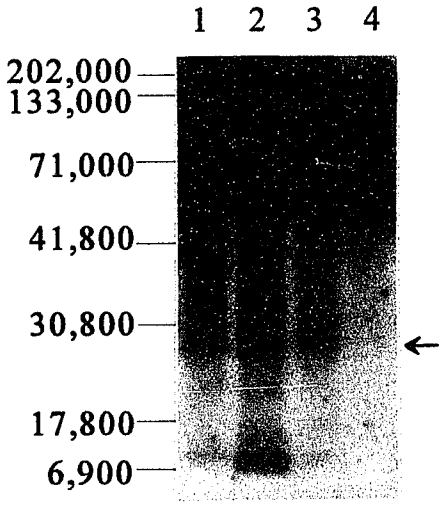

Fig. 2.6. Kinetics of coproantigen appearance measured by WB using a biotin streptavidin modification and TMB substrate. Feces from infected calves at; lane (1) 6 weeks, lane (2) 8 weeks (3), 13 week post-infection and lane (4) pre-infection fecal supernatant.

Table 2.2. Kinetics of the appearance of the 26-28 kD coproantigen in feces of experimentally-infected cattle detected by western blot and compared to the number of eggs .

Number of flukes			Diagnosis at weeks post infection					
			6week		8week		13 wee	
Mat[a]	Imm[b]	Total	Eggs[c]	WB[d]	Eggs	WB	Eggs	WB
38	1	39	0	+++	0	++++	7	+++
25	7	32	0	+++	0	++++	2	+++
26	2	28	0	+++	0	++++	4	+++
22	0	22	0	++	0	+++	3	++
7	3	10	0	+	0	++	0	+

a. Mature flukes.
b. Immature flukes.
c. Eggs per two gram of feces.

DISCUSSION:

The presence of parasite specific antigen in the feces an infected host has recently received considerable attention as a means of diagnosing of a number of parasites (Craft and Nelson, 1982; Green et al., 1995; Rosoff and Stibbs, 1986; Ungar, 1990; Allan et al., 1992; Sirisinha et al., 1991; El-Bahi el al., 1992; Deplazes et al., 1992; Ellis et al.,1993, Deplazes et al.,1994, Espino and Finlay, 1994). The 26-28 kD of *F. hepatica* coproantigen was previously detected using polyclonal antibodies by WB (El-Bahi et al., 1992). In this study I report early detection of coproantigen in subclinically infected cattle using MAbs generated to the 26-28 kD coproantigen. Using WB with the colorimetric substrate TMB, supernatant fluid of these MAbs reacted with the corresponding 26-28 kD band of the ES but were not concentrated enough to reveal the band in the feces. However, the band was detected in the feces using ECL substrate which indicated that the ECL substrate is more senstive than the TMB. By using purified Mabs which contain a higher antibody concentration, the 26-28 kD coproantigen was detected in the feces of animals infected with as few as 22 worms. To further enhance the detection a biotin-streptavidin modification of the WB using TMB and ECLwas developed. Using TMB biotin-streptavidin WB was sensitive enough to detect as few as 10 flukes. The bands were clear with low background. The increased noise associated with the biotin-streptavidin WB and ECL made ECL impractical. I concluded that the most sensitive detection of the coproantigen was achieved using the biotinylated MAbs and TMB substrate, and this procedure was standardized for the remainder of the study.

In a production setting, cattle are often co-infected with other parasites. I wanted to determine if this assay was specific for *F. hepatica*; therefore, FFA and ES of other trematode and cestode parasites that commonly occur in cattle or that are related to *F. hepatica* were used as antigen in WB. I was specifically interested in *P. microbothriodes* because its eggs are sometimes confused with the *F. hepatica* eggs in routine fecal sedimentation examinations. In this assay,I found that the MAbs did not recognize antigens in the ES of *P. microbothriodes.* I believe this test will differentiate between the infection with liver flukes and the rumen fluke. I also did not observe cross-reactivity with the cestode parasite *Moniezia sp.* Minimum cross-reactivity was observed with ES of *F. gigantica* , which is found in tropical and subtropical regions of the world and therefore should not be a problem in North America. The Mabs also cross-react with the ES of *F. magna*, a deer liver fluke that sometimes incidentally infects cattle in ranges shared with deer. However, cattle are an abnormal host, and intense fibrotic encapsulation of *F. magna* typically prevents shedding of eggs into the feces. Further investigation is needed to determine if these antigens are excreted in the feces and if these antigens can be detected in the feces by these MAbs. Espino et al. (1994) detected a coproantigen in the feces of human patients shedding between 25 and 269 *F. hepatica* eggs per gram of feces using a MAb-polyclonal antibody capture ELISA. They found a positive correlation between the *Fasciola*egg count and stool antigen concentration. In the present study, I did not correlate the intensity of the 26-28 coproantigen to the number of eggs; however, this study was short and dealt with low fluke counts. In this study the egg counts were not reliable and

did not indicate the actual number of flukes because immature flukes do not shed eggs (Table 2.2). Unlike the egg counts, I found that the number of flukes and the intensity of reaction in the 26-28 kD coproantigen band were correlated. Using the biotinylated MAbs, the 26-28 kD band was detectable in the feces of infected animals with as few as 10 flukes. Fluke burdens of 20 flukes or less are generally considered to be sub-clinical infections and are often undiagnosed with the fecal egg count procedure in cattle.

Acute fasciolosis is often undiagnosed because *F. hepatica* flukes mature and begin shedding eggs 8 w after infection while the acute symptoms occur earlier (Hillyer, 1986). Prepatent detection of *F. hepatica* infection might be helpful in controlling the disease with drugs directed specifically toward the immature flukes, preventing further damage of the liver parenchyma by migrating flukes. Serological assays have been shown to detect *F. hepatica* as early as 2-4 week post-infection (Zimmerman et al., 1985) but these assays do not necessarily indicate active infection since the antibody level in previously infected hosts can persist after treatment. Moreover, antibodies were not detected in animals infected with less than 27 flukes (Langley and Hillyer, 1989b). Methods for the detection of fasciolosis circulating parasite antigen (Langley and Hillyer, 1989a; Langley and Hillyer, 1989b; Rodrigue-Pérez and Hillyer, 1995) only indicate active infection and could detect the acute subclinical infection; however, these circulating antigen could not be detected once the flukes were established in the bile duct (Langley and Hillyer 1989b). In addition, collection of blood samples for these tests require animal restraint which is often inconvenient especially for herd evaluations. The assay described here detected

coproantigen in the feces of experimentally-infected calves as early as 6 weeks post-infection. Maximum coproantigen concentration was seen at 8 weeks post-infection. This corresponds to the finding of Langley and Hillyer (1989b), and Rodrigue-Pérez and Hillyer (1995) for the detection of the circulating antigen in cattle and sheep. In addition, WB was sufficiently sensitive to detected 10 flukes and collection fecal samples does not require individual animal restraint.

The ability of the WB to detect the antigen before egg shedding suggested that the 26-28 kD coproantigen is not an egg-related antigen. Future studies of the coproantigen will concentrate on localization of the antigen in the flukes, as well as determination of the chemical nature and stability of the coproantigen. Fecal samples used in this study were held longer than 1 year at -20°C, suggesting that the coproantigen is stable. The stability of the coproantigen may make it possible to collect fecal samples directly from the pasture without animal contact, that greatly simplifying herd evaluation proceedures.

CHAPTER III

BIOCHEMICAL CHARACTERIZATION AND LOCALIZATION OF *FASCIOLA HEPATICA* 26-28 kD DIAGNOSTIC COPROANTIGEN

INTRODUCTION:

A variety of antigens are secreted and excreted by parasites and are present in the blood, feces, urine and other fluids of the infected host. These antigens have potential for use in immunodiagnosis and vaccine development. Several diagnostic parasite antigens have been chemically and immunologically characterized. *Schistosoma mansoni* circulating antigens, characterized as polysaccharide by Nash et al. (1974), were shown to be of two types: circulating cathodic antigen (CCA) and circulating anodic antigen (CAA) (Deedler et al., 1976). Both CAA and CCA have been reported to be gut-associated glycoproteins (Nash and Deelder, 1985; Deedler et al., 1985) and several ELISA assays based on the detection of these schistosome circulating antigens have been developed (Deedler, et al., 1989; de Jonge et al., 1990 and Hassan et al., 1992). Among cestode parasites, March et al. (1991) chemically characterized two *Echinococcus granulosus* antigens of serodiagnostic value, an N-glycosylated glycoprotein and a polypeptide. Using MAb, Draelants et al. (1995) characterized an epitope of a circulating antigen with immunodiagnostic value in the ES of *Taenia saginata*. They reported that the epitope is either a carbohydrate or a glycoprotein and that this antigen is present in the tegument of the worm. Studies on the chemical and physical properties of a coproantigen of the

protozoan parasite *Giardia lamblia* by Rosoff and Stibbs (1986) indicated that the coproantigen is a highly glycosylated glycoprotein which is resistant to proteolytic digestion but sensitive to treatment with sodium periodate.

Although diagnostic assays for fasciolosis have been described for detection of either circulatory antigens (Zimmerman et al., 1985; Langley and Hillyer, 1989b; Rodrigue-Pérez and Hillyer, 1995) or coproantigen (El-Bahi et al., 1992; Espino et al., 1994) little is known about the physical and chemical nature of these antigens. The previous chapter described the detection of a 26-28 kD coproantigen using Mabs raised against this particular molecule. Knowledge of the chemical nature, stability and origin of this coproantigen is needed for development of improved immunodiagnostic assays. In this study I biochemically and immunologically characterize the 26-28 kD coproantigen and localize the antigen on mature and immature flukes recovered from bile ducts by immunofluorecence using MAbs.

MATERIAL AND METHODS:

Monoclonal antibodies and Western blot analysis :

The 4 MAbs F10, G7, E7 and F7 were used for the characterization of the coproantigen. The generation of the MAbs and the WB analysis were performed as described in Chapter II.

Excretory-secretory product (ES)

Fasciola hepatica ES product was prepared as described by Santiago and Hillyer (1986) and is reviewed in Chapter II.

Gel filtration chromatography (Sepharose G-75).

The coproantigen was purified from the other ES components using a 0.77x50 cm glass Econo-column (Bio-Rad Laboratories Inc.) packed to a bed height of 40 cm with Sephadex G-75 superfine beads (Pharmacia Biotech Inc, Picataway, NJ). The column was calibrated using a Pharmacia Gel Filtration Kit for molecular weight determination of low molecular weight proteins (Pharmacia Biotech Inc.). PBS was used for the fractionation of *F. hepatica* ES product. Four mg of concentrated ES in 1 ml PBS were applied to the column followed by 1ml of 1% methyl green (Sigma Chemical Co., St.Louis, MO) in PBS. The complete Econo System (Bio-Rad Laboratories, Inc.) was used in the fractionation at a flow rate of 22 ml/h. Fractions were collected in 0.5 ml volumes and were examined for the presence of the coproantigen by WB.

Differential staining:

The chromatographically purified 26-28 kD antigen was diluted in sample buffer under reducing and non-reducing conditions. All samples were boiled at 100°C for 5 m, then separated on a 12% acrylamide SDS-PAGE gel by the method of Laemmli (1970). Gels were then stained with: 1) Coomassie blue stain (Sigma Chemical Co.); 2) silver stain (Bio-Rad laboratories Inc.); 3) periodic acid Schiff reagent stain (PAS; Polyscientific Research and Development Corp., Bay Shore, NY.) and 4) PAS silver stain. Coomassie

staining was done according to the method of Hames 1990. A Bio-Rad Silver Stain Kit was used for silver staining of the gels according to the manufacturer's instructions for staining 0.5-0.1 mm gels. The PAS staining was performed according to the method of Jay et al. (1990). Briefly, gels were fixed in 50% (v/v) methanol with gentle agitation, incubated for 20 min in distilled water, and then incubated in 2% sodium periodate (Sigma Chemical Co.) for 15 min. After two 2 min washes with distilled water, concentrated Schiff reagent was added and the gels were gently agitated in a fume hood for 2 hr to overnight. The gels were destained by washing in several changes of distilled water until the background faded and the bands appeared magenta in color. PAS-silver staining was done according to the method of Jay et al.(1990). Briefly, after staining with PAS, the gels were washed twice with distilled water and reduced with 2% (w/v) sodium metabisulfate (Sigma Chemical Co.) in distilled water overnight. The gels were then washed for 30 min with several changes of distilled water until the water remained clear and then silver stained as described above.

Alkaline treatment and enzymatic deglycosylation:

Purified coproantigen was brought to pH 10 using 0.1M NaOH and incubated in the dark overnight at 37°C . The pH was neutralized by the addition of 0.1 M HCl prior to analysis by WB. Enzymatic deglycosylation was performed on the purified antigen by using endoglycosidase F and endoglycosidase H (EndoF and EndoH; Boehringer Mannheim Biochemica. GmbH, Germany) as described by the manufacturer. For Endo F treatment, 4μg of chromatographically purified 26-28 kD antigen was diluted 1:5 (v/v)

in 100mM sodium phosphate, 25mM EDTA, 5mM sodium azide and 50%, v/v, glycerol, pH 7.2 and boiled for 1 min. To 90 µl of the mixture, 0.5% Triton X 100, 1% 2-ME and 1 U of EndoF were added. After an 18 hr incubation at 37°C an additional 0.4 U of Endo F were added and incubated for an additional 3 hr. For Endo H treatment, purified 26-28 kD antigen was diluted 1:5 (v/v) 50 mM sodium phosphate buffer, 25mM EDTA, 0.05% (w/v) sodium azides pH 7 boiled then boiled for 1 min. To the mixture, 1% triton X 100 and 7% 2-ME were added, followed by the addition of 10 µU of Endo H. After 18 hr incubation at 37°C, an additional 2 µU of EndoH were added and incubated for an additional 3 hours. Purified antigen treated as above but without the addition of the enzymes served as controls.

Ion exchange chromatography:

Excretory secretory product components were separated using a 0.77x50 cm glass Econo-column (Bio-Rad Laboratories, Inc.) packed to a bed height of 20 cm with Q Sepharose Fast Flow (Pharmacia Biotech Inc. , Picataway, NJ). Four milligrams of ES protein in 1 ml 25 mM NaCl was applied to the column. Gradient elution was performed to release bound molecules using NaCl at molarities of 25mM to 500mM. Fractionation was performed using the Complete Econo System (Bio-Rad) at a flow rate of 2 ml/min. Collected fractions were tested for the presence of the antigen by WB.

Gelatin SDS-PAGE:

The method of Dalton and Hefferman (1989) was used. Briefly10µg of purified 26-28 kD coproantigen was applied to a 12% acrylamide gel with the following

modification: both the stacking and separating gels contained 0.1% bovine gelatin (Sigma Chemical Co.). Ten units pronase E and 10 U trypsin (Sigma Chemical Co.) were used as controls. Gels were washed for 1 hr in the incubation buffer (see below) containing 2.5% Triton X-100 with one change, then incubated in the incubation buffer alone for 16 hour . Four different incubation buffers were tested: 0.1 M glycine, pH 8.0; 0.1 M glycine pH 7.0; 0.1M sodium citrate, pH 4.5 and 0.1M sodium citrate, pH 3.0. Following incubation, gels were stained with 0.04% Coomassie brilliant blue dissolved in stacking solution (20% methanol: 10% acetic acid: 70% water) and destained in the same stacking solution.

Protease digestion:

The proteolytic sensitivity of the coproantigen was determined by incubation with pepsin, trypsin and pronase E (Sigma Chemical Co.) according to Rosoff and Stibbs (1986). Pepsin and trypsin were used at a concentration of 2000 U/ml in 0.1 M Tris hydrochloride, pH 8.0. The 26-28 kD protein was digested for 24 hours at 37°C. Pronase E was used at a concentration of 100 U/ml in 0.1 M Tris hydrochloride, pH 8.0 containing 1 mM $CaCl_2$. The purified 26- 28 kD protein was incubated for 15 min at 37°C followed by digestion at 60°C for 24 h. As a control, the 26-28 kD protein was incubated in 0.1 M Tris hydrochloride, pH 8.0 alone. After digestion all the samples were evaluated by SDS-PAGE and WB as described above.

The effect of temperature on the antigen :

To determine the effect of temperature on the antigenicity of the coproantigen, the 26-28 kD antigen was incubated for 15 min in a heat block at 100°C or overnight, for 3

days or for 1wk at room temperature and incubated for up to 2 month at 4°C. All the incubations were terminated by boiling the antigen in SDS-PAGE sample buffer, and the effect of these incubations were evaluated by SDS-PAGE and WB.

Indirect immunofluoresence :

The four MAbs (F10, G7, E7 and F7) specific for the 26-28 kD coproantigen were used to localize the coproantigen on cross sections of the flukes. Murine IgM, κ Protein Control (Hyclone Laboratory Inc., Logan,UT) was used as a control. For preparation of the fluke sections, living flukes were collected from the bile ducts of infected cattle at necropsy and placed into RPMI media. Flukes were frozen by Cryokwik (International Equipment Company, Needham height, MA.). Frozen cross sections of the flukes were prepared and later fixed in acetone: ethanol solution (1:1) for 5 min at - 20°C. Slides were washed twice with PBS. Each of the four MAbs and the mouse IgM, κ protein control were diluted separately to 1μg/ml in PBS contains 1% bovine serum albumin (Sigma Chemical Co.) and 5% normal goat serum (Sigma Chemical Co.; dilution buffer). The fixed slides were immersed in the diluted MAbs for 1 hr at room temperature with gentle shaking. The slides were then rinsed with PBS followed by a 10 min wash with PBS with gentle shaking. The sections were then immersed in a 1:100 dilution of DTAF-labeled anti-mouse IgG (Jackson Immuno Research Laboratories, Inc., West Grove, PA.) in the dilution buffer and incubated with gentle agitation for 1 hr. Sections were washed as above and counter stained with 0.1% Evan's blue (Sigma Chemical Co.) in PBS for 5 min, washed with distilled H_2O and mounted with 0.5% glycerol in PBS prior to examination.

RESULTS:

Purification of F.hepatica antigen by gel filtration:

Because the coproantigen is found in low concentration in the feces and feces is highly contaminated with organic materials, the MAbs recognize the 26-28 kD molecule in the ES by WB and the molecule is present at relatively high concentrationin ES; therefore, I choose to used *F. hepatica* ES as a source of the 26-28 kD coproantigen for the biochemical studies. I was unable in several attempts to purify the 26-28 antigen from ES by affinity chromatography using the MAbs; therefore, I purified the coproantigen by gel filtration using G-75 superfine column. When the concentrated ES was fractionated on the calibrated column, two major peaks and one small peak were resolved. The purity of the fractions was analyzed by SDS-PAGE. The first peak was the void volume which contain the higher molecular weight proteins. The only band that was detected in the second peak was the 26-28 kD antigen and this antigen was recognized by the MAbs using WB (Fig. 3.1). Comparing the peaks from ES to those of the calibration kit, the coproantigen peak was found between the 25 kD peak and 13 kD peak with a predicted MW by chromatography of 18 kD (Fig. 3.2).

Differential staining of coproantigen:

Since different molecules have different staining properties, the staining properties of the coproantigen in ES were investigated. Results are shown in Fig 3.3. When gels were stained with coomassie blue, the major stained band was the 26-28 kD band. When stained

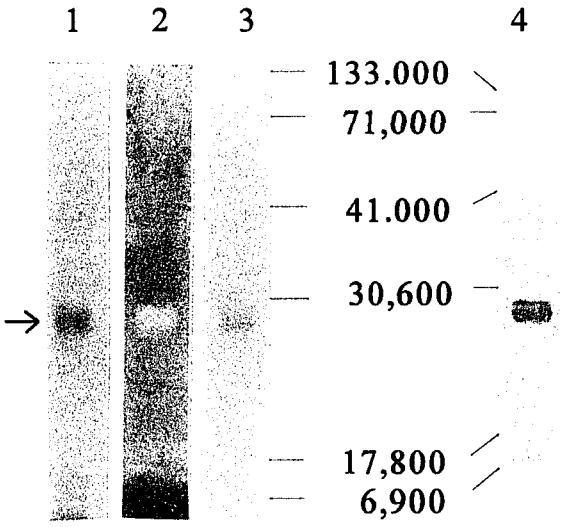

Fig. 3.1. Differential stains and western blot analysis of chromatography purified 26-28 kD antigen; lane (1) SDS-PAGE gel stained with Commassie blue, lane (2) silver stain, lane (3) PAS, and lane (4) reacted with the specific Mab (F10) in WB.

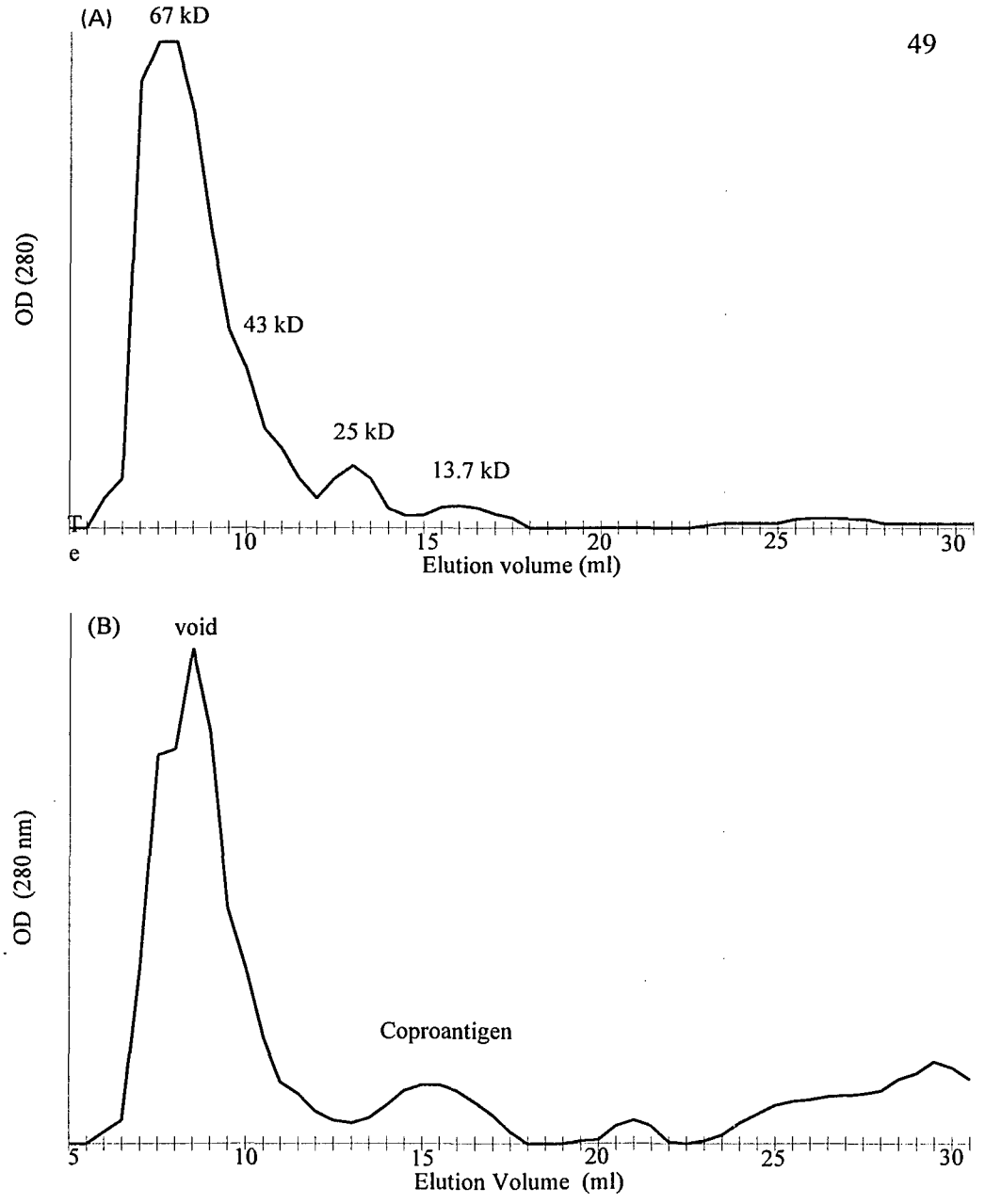

Fig. 3.2. Sephadex G-75 (superfine) gel filtration elution profile of the 26-28 kD antigen from ES. (A) Calibration of the column with commercial protein standards and (B) *F. hepatica* ES.

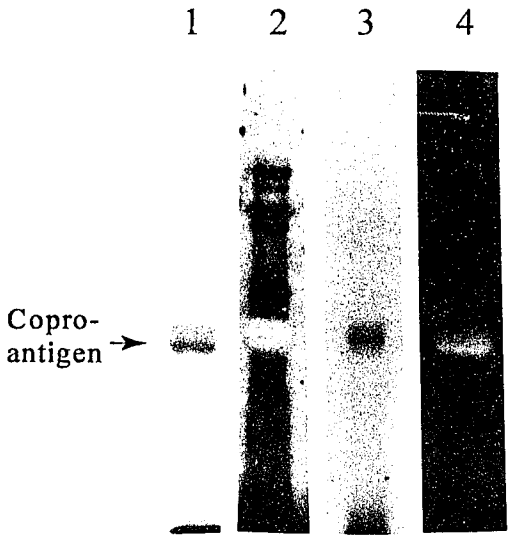

Fig. 3.3. Differential staining of SDS-PAGE separated ES of *F. hepatica* showing the 26-28 kD coproantigen. Lane (1) stained with commassie blue, lane (2) stained with silver stain, lane (3) stained with PAS stain and lane (4) stained with PAS-silver stain.

with silver stain, other proteins found in ES product were stained and the 26-28 kD antigen was negatively stained which suggested that the antigen might be a glycoprotein. To confirm the silver stain results, the PAS staining profile of the coproantigen was investigated. I found that the band stained clearly with PAS indicating that the molecule was a glycoprotein. A PAS-silver stain, which stains glycoproteins but not proteoglycan (Jay et al., 1990), was then performed and the 26-28 kD band remained unstained. There were no differences between the size of the band under reducing and non-reducing conditions (Fig 3.4). Results were confirmed by similar differential staining of the purified 26-28 kD coproantigen. These results indicated that the coproantigen is a monomeric, highly glycosylated glycoprotein, and most probably it is a proteoglycan.

Alkaline treatment and enzymatic deglycosylation:

Since the differential staining indicated the coproantigen molecule is a proteoglycan, attempts were made to identify the type of the glycosylation. Alkaline treatment of the coproantigen resulted in the disappearance of the 26-28 kD band and appearance of a new band with a MW of 8 kD in a coomassie blue stained gel. The 8 kD band was recognized by all four MAbs on WB, a representative WB is shown in Fig. 3.5. Neither Endo F nor Endo H had an effect on the size or the antigenicity of the 26-28 kD band (Fig 3.5). These results suggest that the coproantigen is an O-glycosylated protein in which 70% of its size is a carbohydrate. Further the specific epitope recognized by the MAbs is on the protein core, not in the carbohydrate component of the molecule. .

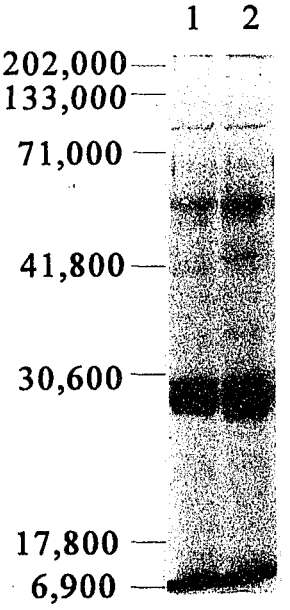

Fig. 3.4. Commassie blue stain ofthe 26-28 kD coproantigen on SDS-PAGE under reducing and non-reducing conditions. Lane (1) reducing condition and lane (2) non-reducing condition.

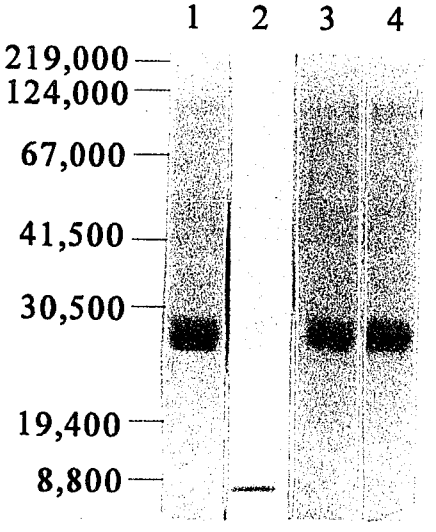

Fig. 3.5. The effect of the alkaline treatment and enzymatic deglycosylation on the coproantigen as detected by the specific MAb F10 by WB. Lane (1) untreated, lane (2) alkaline-treated, lane (3) Endo F-treated and lane (4) Endo H-treated 26-28 kD antigen.

Charge of the coproantigen:

To determine the charge of the coproantigen, Q Sepharose Fast Flow Anion exchange chromatography column was used. The results are shown in Fig. 3.6. While the major part of the 26-28 kD antigen was eluted with the void volume at 25 mM NaCl, a portion of the coproantigen was later eluted in at 250-375 mM NaCl . These results indicate differences in the charge of the coproantigen which could be attributed to the differences in glycosylation.

Protease activity of the coproantigen:

Many previous studies reported proteolytic enzymes in the products released by tissue penetrating parasites. To determine if the 26-28 kD coproantigen plays any role in penetration of the liver parenchyma, the protease activity of the purified antigen was analyzed by gelatin SDS-PAGE . After electrophoresis, gels were incubated individually at pH 8.0, 7.0, 4.5 or 3.0. No protease activity was detected for the coproantigen at any pH, while at pH 7.0 and 8.0 both protease and trypsin digested the gelatin, resulting in clear areas when stained with Coomassie blue (Fig 3.7).

Sensitivity of coproantigen to the proteolytic enzymes:

The coproantigen characterized in this study is secreted and excreted by the flukes in the bile duct. This protein moves through the digestive tract where it is subjected to different digestive processes, including proteases. The sensitivity of the coproantigen to pepsin, trypsin and pronase E was examined. The purified 26-28 kD protein was digested with enzymes and analysed by SDS-PAGE and WB. While the purified coproantigen was

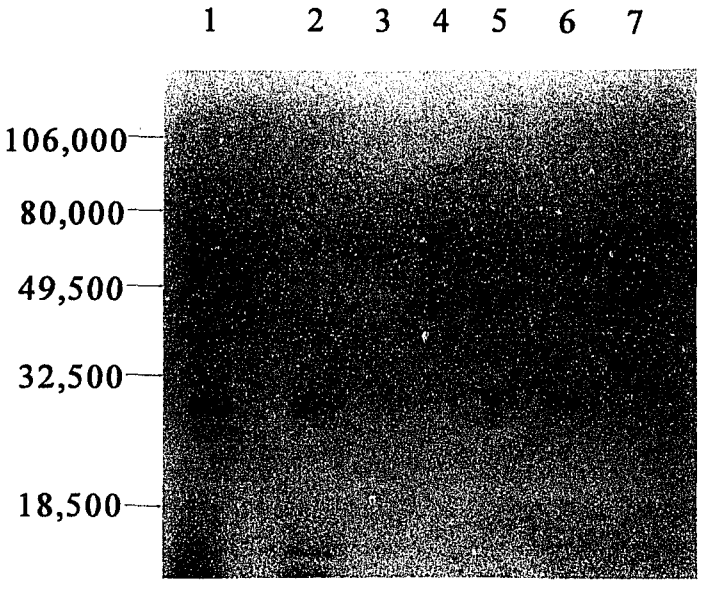

Fig. 3.6. Western blot analysis of fractions from ion exchange chromatography of the ES using Q Sepharose. Lane (1) unseparated ES as a control. Fractions collected at: lane (2) 25 mM, lane (3) 50 mM, lane (4) 125 mM, lane (5) 250 mM, lane (6) 375 mM and lane(7) 500 mM.

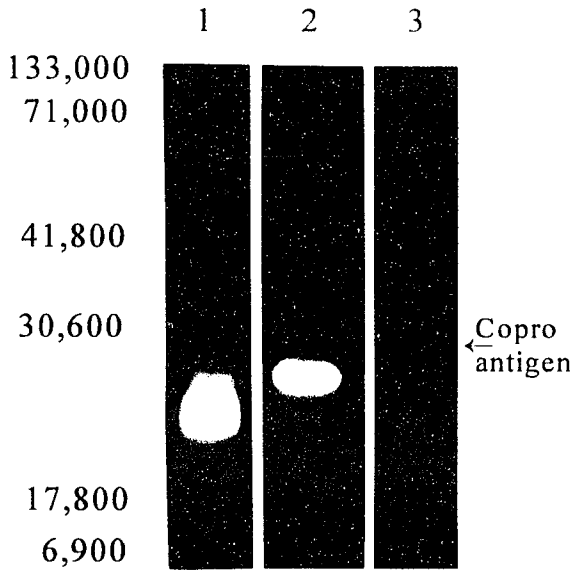

Fig. 3.7. Determination of protease activity of the 26-28 kD antigen using Gelatin SDS-PAGE, stained with Commassie blue. Lane (1) trypsin, lane (2) pronase E as controls, and lane (3) is the 26-28 kD antigen.

not affected by the proteolytic activity of pepsin and retained its reactivity with the specific MAbs, the coproantigen was sensitive to pronase E and trypsin. After digestion with pronase E no band could be detected by WB indicating that pronase E destroyed the reactive epitope. After partial digestion with trypsin the 26-28 kD band became fainter in WB and another molecule was created that was recognized by the MAbs. The new molecule had a molecular weight of 18 kD (Fig 3.8). The 26-28 kD molecule in ES was also resistant to treatment with pepsin and degraded by pronase E, however, after treatment with trypsin, the MAbs recognized two bands: the first was the 18 kD band, and the second is a new 40 kD band (Fig. 3.8). These result indicate that the antigenicity of the coproantigen is not affected by pepsin or trypsin but it is sensitive to pronase E which confirms the protein nature of the epitope. Further it indicates that the epitope is created or exposed on a larger molecule following treatment with trypsin.

The effect of temperature:

Since the 26-28 kD antigen detected in feces will be used in a diagnostic test for *F. hepatica*, attempts were made to determine the stability of the coproantigen. Boiling of the 26-28 kD coproantigen has no effect on the size and antigenicity of the molecule (data not shown). The effect of temperature on coproantigen was evaluated by SDS-PAGE followed by Coomassie stain and by WB against the specific MAbs (Fig. 3.9). Overnight incubation of the antigen at room temperature gave rise to several new smaller MW bands ranging from 20- 8 kD and the incubation of the antigen at 4°C for 2 month generated a smaller band around 20 kD; this band decreased in size after longer incubation. All of the

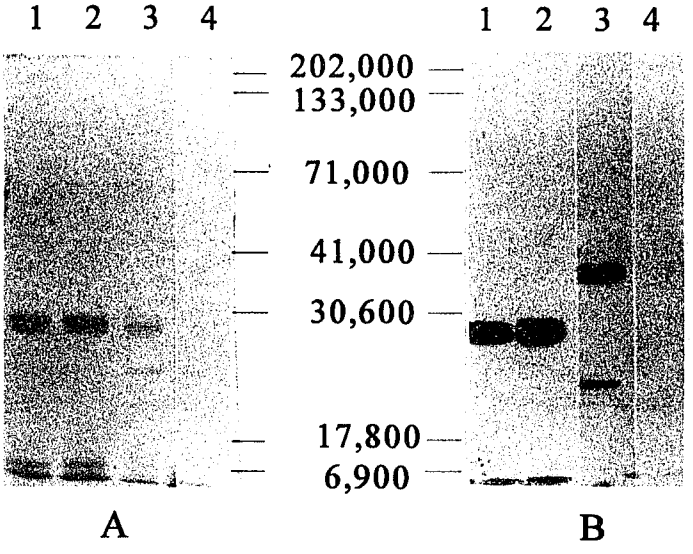

Fig. 3.8. The effect of protease treatment on purified 26-28 kD antigen and ES as detectedusing specific MAb F10 in WB. Protease treatment of (A) purified 26-28 kD antigen and (B) *F. Hepatica* ES. Lane (1) untreated coproantigen, lane (2) pepsin digested coproantigen, lane (3) trypsin digested coproantigen, and lane (4) pronase E digested coproantigen.

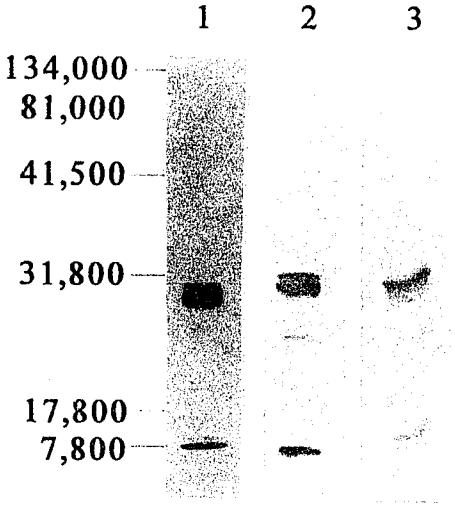

Fig. 3.9. The effect of different storage temperature on stability of coproantigen as detected usingMAb F10 in WB. The 26-28 kD antigen stored lane (1) more than 3 years at -20°C, lane (2) for 2 months at 4°C and lane (3) overnight at room temprature.

new bands were recognizable by the MAbs. From these results, I concluded that the coproantigen was relatively stable at different storage temperatures and even when the molecule was degraded antigenicity was maintained.

Localization of coproantigen on the adult fluke:

Using three of the MAbs (F10, G7 and E7) separately as the primary antibody in an indirect immunofluorescent antibody assay on frozen sections of the adult worms, a strong fluorescence was observed in the tegument and the gut. For the MAb F7, florescence was noted only in the gut of the adult worm. No florescence was observed for the mouse IgM protein that was used as a control (Fig. 3.10). These results suggests that the source of the coproantigen is present in the tegument and the gut of the flukes.

DISCUSSION:

A 26-28 kD coproantigen was detected in the feces on of the cattle using polyclonal antibody or MAbs in WB (El-Bahi et al.1992), but the biochemical nature of the antigen was unknown. Characterization of the nature of this antigen, where it is produced, and its stability to environmental and enzymatic degradation, are important for development of an effective diagnostic test. The 26-28 kD antigen is highly diluted in the feces and would be difficult to purify from other organic and non-organic materials contained withen the feces; therefore I chose to work with *F. hepatica* ES which contains a molecule of the same molecular weight and antigenicity as the coproantigen and is available at higher concentration.

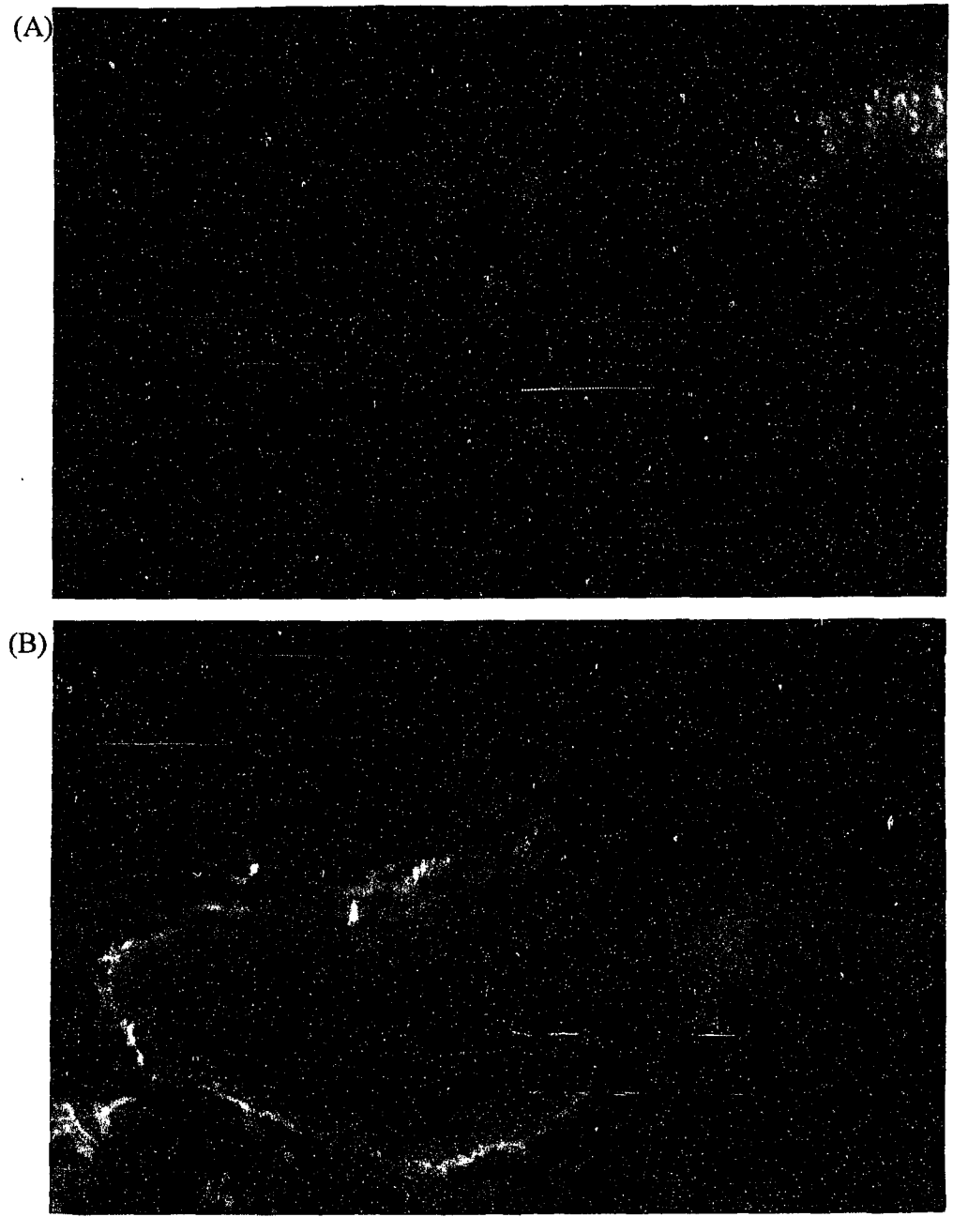

Fig. 3.10. Localization of the coproantigen on cross sections of mature *F. hepatica* using indirect immunofluorescent antibody. Panel (A) the tegument and panel (B) the gut of the fluke x100.

Several attempts were made to purify the 26-28 kD antigen using the MAbs but were unsuccessful. The low affinity of the IgM MAbs might be the reason; however, the four MAbs were noted to bind with higher affinity to the denatured molecule than to the native molecule which may explain the problems with the affinity purification. Therefore, the 26-28 kD antigen was purified by gel filtration. Comparing the antigen peak to the gel filtration molecular weight standards I estimated that antigen had a molecular weight of 18 kD (Fig 3.1) while its molecular weight was estimated at 26-28 kD by SDS-PAGE. Gel filtration is a poor method for estimating the molecular weight of glycoproteins containing more than a few percent of carbohydrate due to their asymmetric branched structure (Beely, 1985). These results suggest that the antigen is a compact molecule which behaves as 18 kD protein on gel filtration. Further, biochemical analysis of the 26-28 kD antigen under reducing and non-reducing conditions, indicated that the coproantigen exists as a monomer.

Little or no silver staining is observed for those glycosylated proteins of which 50% or more of their molecular weight is carbohydrate, most probably due to stearic interference of the carbohydrate with the binding with silver (Jay et al.,1990). The pattern of staining of the 26-28 kD coproantigen with Coomassie blue and PAS stains and the negative staining with silver stain indicated that the molecule is a highly glycosylated protein. The PAS-silver stain is a sensitive method for the staining of glycoprotein, however, it fails to stain proteoglycan (Jay et al., 1990). The failure of the 26-28 kD band to stain with PAS-silver stain suggests that the molecule is a proteoglycan. After identifying the molecule as a proteoglycan, I examined the type of glycosylation. The release of

carbohydrate units by alkali has been most widely applied to glycoproteins and proteoglycans containing alkali-sensitive O-glycosidic linkages. At high pH, the O-glucosidic linkage between glycan and *B*-hydroxyamino acid of serine and threonine are cleaved (Beely 1995). When the pH of the purified coproantigen was increased to pH 10 the 26-28 kD band was no longer found in the stained gel and could not be detected in WB. Concurrently, a smaller band of MW of 8 kD appeared and was recognized by the specific MAbs. This indicated that the coproantigen might be O-glycosylated. However, other workers have shown that even the mild alkaline treatment of glycoproteins or proteoglycans under conditions severe enough to release O-linked oligosaccharide will also liberate many N-linked glycans. To eliminate the possibility of being N-linked, the coproantigen was subjected to enzymatic deglycosylation using EndoF and EndoH. These enzymes act on the asparagine-linked carbohydrate groups of glycoprotein. The coproantigen was stable under these enzymatic deglycosylations as described here; However, since 70% of the MW of the antigen is carbohydrate, it is possible that the large carbohydrate moieties prevented the from reaching their cleavage sites. Due to variability in glycosylation, glycoproteins may show evidence of polydispersity of molecular weight, which result in broad peaks in gel filtration elution profiles and a broad bands on SDS-PAGE (Beely 1985). The 26-28 kD antigen gave this characteristic polydisperse pattern, and gave different peaks based on charge on the ion exchange chromatography.

Like many other helminthic parasites that penetrate the tissue of the host by the action of the proteolytic enzymes, *F. hepatica* releases enzymes that help the parasite in its migration

from the intestine to the liver where it continues to migrate until it reaches its final habitat, the bile duct. Dalton et al. (1989) identified 11 distinct proteases in *in-vitro* cultures of *F. hepatica* that play a role in degradation of cells and proteins and penetration of host tissue. Two of these proteins had a molecular weight in the 26-28 kD range (28 and 27.5 kD). To determine if the 26-28 kD coproantigen identified by the MAbs is a protease, the SDS-gelatin method of Dalton et al. (1989) was applied to the coproantigen. Results indicated that the coproantigen is not a protease and that this molecule is unique.

In transit from the bile ducts through the intestinal tract, the coproantigen is exposed to digestive enzymes such as pepsin (found in the gastric juice) and trypsin (found in the small intestinal digestive fluid), therefore I wanted to determine the effect of these proteases on the coproantigen. As with other proteins, prolonged pronase E digestion of the coproantigen resulted in very extensive degradation of the peptide chain of the glycoproteins, which resulted in the disappearance of the coproantigen band from the stained gel and WB. Results of this study showed that the 26-28 kD antigen is not digested by pepsin, which cleaves peptide bonds containing phenylalanine, tryptophane, or tyrosine. However, it was digested by trypsin, which cleaves peptide bonds containing lysine or arginine. At least three cleavage sites were detected as indicated by the disappearance of the 26-28 kD band and the creation of four new bands of lower molecular weight. The MAbs detected one of the new bands at MW of 18 kD, which indicated that trypsin cleaved the molecule without altering the epitope recognized by the specific antibody. On the other hand, trypsin treatment of the ES resulted in the detection of the 18 kD band and another band of higher molecular weight (40 kD)

. These results suggest that trypsin may have cleaved a previously unrecognized higher MW molecule to create a smaller molecule that is recognized by the specific MAbs.

Since the overall goal of this work is to develop a diagnostic test for detection of coproantigen, another important factor which I wanted to address was the stability of coproantigen under transport and storage conditions that typically occur when samples are submitted to diagnostic laboratories for egg detection. Studies on the effect of different temperatures on the coproantigen revealed that although there are shifts in the band size due to either, gradual loss of glycosylation or proteolytic cleavage, antigenicity was retained when the ES was left overnight at room temperature, for two months at 4°C and for more than three years at -20°C. The same results were obtained using stored fecal samples (data not shown) where I observed a shift in the band size after storage 3 years at -20°C. I also detected several bands from the stored feces by WB that ranged from the 28 to 8 kD.

Finally I wanted to determine the location of the coproantigen on the flukes themselves using the specific MAbs. The tegument of the flukes consists of two main layers, the distal and the proximal cytoplasm. The outer zone or the distal cytoplasm where the spines are embedded is coated by the glycocalyx which is sloughed continuously. The proximal cytoplasm contains tegumental cells (T1 and/or T2). These cells are responsible for maintaining the glycocalyx. Proximal and distal cytoplasm are connected by tubules underlying these tegumental cells and the translocation of the content of these cells to the distal cytoplasm takes place through these connecting tubules. In the newly excysted juvenile the tegumental cells are called T0 cells. During migration to the liver the T0 cells metamorphose into T1 cells which

secret T1 granules. Upon entering the bile duct as immature flukes, T2 cells start to appear and for a transitional period the glycocalyx will be formed by both the T1 and T2 granules, followed by a decrease in the number of T1 cells (reviewed by Reddington et al., 1984). Using IFA, I localized the antigen to the cells of the proximal layer of the tegument. Since studies were conducted on mature flukes, the source of the coproantigen is most probably the T2 cells. The other possible source of the coproantigen is the gut lining of the flukes. Hanna and Trudgett (1983) raised MAbs against the tegumental antigen present in T1 granules and glycocalyx of the flukes. In addition to labeling the T1 cells and glycocalyx, these MAbs labeled the gut and excretory system of the flukes which indicated that these internal structures have antigenicity in common with the tegument. My findings are similar in that I localized the antigen to the tegument and the gut of the flukes. In schistosomiasis, the major circulating antigens are reported to be gut associated glycoprotein and proteoglycans (de Jonge et al., 1990). While methods for detection of circulating antigens of *Schistosoma* rely on the detection of the carbohydrate epitopes of the gut-associated glycoproteins and proteoglycans (Nash et al.,1974; Deelder et al., 1980; de Jonge et al., 1988; Deelder et al., 1989; de Jonge et al., 1990), results on *F. hepatica* reported here indicate that these MAbs detect the protein core of similar antigens.

In summary, this work has demonstrated that: 1) the 26-28 kD antigen is a monomeric proteoglycan which is secreted and excreted from the tegument and the gut of the flukes; 2) the antigenicity of the coproantigen is stable or even enhanced by the action of proteolytic enzymes found in the digestive tract and 3) the antigenicity of the coproantigen is stable

under a variety of standard laboratory storage conditions, suggesting that fresh fecal samples can be collected from the pasture for herd evaluation without animal restraint and handling. The stability of coproantigen antigenicity may be due to its highly glycosylated nature which may protect the protein core. These factors support the use of the 26-28 kD coproantigen in an immunodiagnostic test for fasciolosis.

CHAPTER IV

EVALUATION OF A MONOCLONAL ANTIBODY-BASED CAPTURE ELISA FOR THE DETECTION OF *FASCIOLA* 26-28 kD DIAGNOSTIC COPROANTIGEN IN THE FECES OF CATTLE.

INTRODUCTION:

Diagnosis of *Fasciola hepatica*, an economically important parasite of domestic animals, is usually achieved by identifying eggs in the feces. However, by the time eggs are produced severe damage in the liver parenchyma could have been occurred. Thus, more sensitive diagnostic methods would be very useful for the diagnosis of fasciolosis. Two types of immunoassay have been developed in an effort toward this goal. One approach focused on the detection of antibodies in the serum of infected animals (Farrell et al., 1981; Hillyer et al., 1981, Hillyer et al., 1985; Zimmerman el al., 1982; Zimmerman et al., 1985; Welch et al., 1987 and Hillyer and Soler de Galanes, 1991). These serological tests succeeded in detecting *F. hepatica* infection as early as 2-4 weeks post-infection, an advantage over the current sedimentation techniques in which worms are not detected before they mature and begin egg shedding, approximately 8 weeks after infection. The second approach relied on the detection of the circulating parasite antigens. These assays have the advantage of detecting only active infections. Circulating antigens can be detected in infected humans, mice, cattle and sheep using ELISA (Espino et al., 1990; Langley and Hillyer, 1989a; Langley and Hillyer, 1989b; Rodrigue-Pérez and Hillyer, 1995). While circulating antigens were

detected as early as 4-6 weeks post-infection (Rodrigue-Pérez and Hillyer, 1995), the detection of circulating antigen has some disadvantages: 1) immunocomplex formation tends to reduce the number of epitopes available for the tests and so decreases the sensitivity (Langley and Hillyer, 1989a); 2) once the flukes enter the bile duct their various antigenic products are no longer available in the circulation (Hanna, 1980b), and 3) since it requires serum, sampling of large herds is difficult.

Detection of *F. hepatica* antigens in the feces of the infected host might be a useful and convenient method for diagnosis of fasciolosis and this is support by several studies. Youssef et al. (1991) detected a coproantigen of *Fasciola gigantica* in human feces using counterimmunoelectrophoresis. Using WB, El-Bahi et al.(1992) detected a 26-28 kD coproantigen of *F. hepatica* in the feces of naturally infected cattle. Espino and Finlay (1994) reported the detection of a coproantigen using ELISA from feces of people infected with *F. hepatica*. Using WB, I detected the 26-28 kD coproantigen in experimentally-infected calves using MAbs (Chapter II). Coproantigen detection has advantages over the serologic assays in that only the active infections are detected, there is no possibility of immunocomplex formation and animal handling is not required to obtain samples scince fresh feces can be collected from pastures.

I have demonstrated that the 26-28 kD coproantigen is useful in diagnosis of fasciolosis (Chapter II) and that the 26-28 kD coproantigen possesses the characteristics of a good diagnostic coproantigen; the coproantigen is stable in the host gastrointestinal tract and under different storage temperature (Chapter III). The antigen is shed by mature and immature

flukes in the bile duct, making early detection possible, and it is present in detectable quantities. However, the WB is a technically complicated assay. In the current study, specificMAbs (F10) raised against the coproantigen was used to develop a capture ELISA for the detection of mature and immature liver stages fasciolosis.

MATERIAL AND METHODS:

Antibodies:

MAb M2 DS/DSF10 (F10) specific for the 26-28 kD coproantigen was prepared as described previously (Chapter II). P3.2.6.1 myeloma was purchased from ATCC.Ascites fluid was produced from the myeloma according to the method of Gallate (1987).

Rabbit anti-26-28 kD coproantigen was obtained by immunizing two female 7-8 lb New Zealand white rabbits with the chromatographically purified 26-28 kD coproantigen (Chapter III). Two hundred micrograms of coproantigen was emulsified with 0.5 ml of Titer Max adjuvant (Vaxcel, Inc., Norcross, GA). For each injection, rabbits were hyperimmunized by several subcutaneous and intramuscular inoculations on the dorsal aspect and the hind quarter of the rabbit, followed by two similar injections at 15 day intervals. Blood was collected 7-15 days after the final injection. Serum was pooled and stored at - 20 °C.

Calves:

Yearling cattle were obtained from a fluke free-area and confirmed to be fluke-free by fecal egg count using a sieve-sedimentation procedure (Flukefinder, Moscow, ID). Each animal was experimentally infected with 500 metacercaria. Feces were collected at 6, 8,

10, and 13 weeks post-infection. Animals were slaughtered 14 weeks after infection, and the number of flukes recovered from each animal was determined by the method of Malone et al.(1982).

Excretory secretory product and fecal sample preparation:

Excretory secretory product of *F. hepatica* was prepared as described by Santiago and Hillyer (1986) and is reviewed in Chapter II. Fecal egg counts and fecal supernate preparation were preformed as described in Chapter II.

Antigen capture ELISA protocol:

The assay was optimized using the ES product of *F. hepatica* which contains the coproantigen and the ES of *Paramphistomum microbothroides* which is not recognized by MAb F10 (Chapter II). The optimal dilution of the polyclonal antibody and the enzyme conjugate were determined by checker board titration using negative and positive fecal supernatant. The optimum dilution of the polyclonal serum was 1:50 and of the goat anti-rabbit IgG horseradish peroxidase conjugate was 1: 500. Flat bottom 96-well Immulon 4 microtiter strips (Dynatech Laboratories, Chantilly, VA) were sensitized overnight with 1.5 µg/well saturated ammonium sulphate-purified MAb F10 diluted in 0.1M carbonate buffer, pH 9.6. Strips were incubated at 4°C overnight; afterward the wells were washed 6 times with 0.05M Tris, 0.001M EDTA and 0.15 NaCl (NET) containing 0.1% Tween 20 (NET-T). Wells were blocked with 200 µl of the NET containing 3% fish gelatin and 0.2% 1M sodium azides (blocking solution). After incubation at room temperature for two hours, wells were washed as before and stored at -20°C until use. Fecal supernatant were diluted 1:2 in NET-T contain

3% fish gelatin and 1% mouse myeloma ascites (dilution buffer), and 100 µl were added to the wells in triplicates. The strips were incubated for 1 hr at room temperature then washed 8 times as before. One hundred microliters of rabbit anti-26-28 kD coproantigen serum diluted 1:50 in dilution buffer, were added to each well. The strips were then incubated for 2 hr at room temperature. After incubation, wells were then washed 6 times as before and 100µl of peroxidase-conjugated goat anti-rabbit IgG (H+L) chain (Cappel Laboratories, Cochranville, PA.) diluted at 1:500 in the dilution buffer, was added to each well. After incubation for one hour, wells were washed 6 times as before and 100 µl of the TMB substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) were added to each well. The reaction was stopped by the addition of 50 µl 0.1 M sulfuric acid. Color change was measured at 490 nm using Dynatech MR 5000 microplate reader (Dynatech Laboratories). ES of *F. hepatica*, ES of *P. microbothroides* and the blocking solution were always included as controls.

To determine the sensitivity of the test, 40 µg of the chromatographically-purified 26-28 kD coproantigen were diluted in a negative fecal supernatant.

Statistical analysis:

Each assay was done in triplicate. Absorbance readings from wells with the same sample were expressed as an average. Inter-assay variation was determined by repeating the assay four times on four different days. The sample was defined as positive if the optical density (OD) value exceeded the mean of the negative control feces (pre-infection) plus 2standard deviations. The difference between the OD values of the pre-infection and post-infection of each fecal sample was calculated and tested using the one-tail paired *t* test.

RESULTS:

Coproantigen detection:

The fecal capture ELISA for coproantigen was used to detect the antigen in the fecal supernatant of 27 experimentally infected calves with flukes burdens ranging from 1 -39. Pre-infection fecal supernatant were also tested. The post-infection ELISA ODs were significantly greater than the pre-infection ODs (one-tailed paired *t*-test p, 0.05) and mean from infected cattle feces were significantly different from those of control feces (Fig. 4.1). In addition, there was an apparent correlation of fluke number with OD values. (Fig. 4.2).

Idefined a positive fecal sample as greater than the mean of the negative controls plus 2 standard deviation. Using this criteria for positivity, I detected *F. hepatica* in infected animals with 10 flukes or more. All animals with less than 10 flukes were considered as negative using the criteria of this test. The inter-assay variation and the intra-assay variation was determined and the results are illustrated in (Table 4.1)

Sensitivity of the test:

To determine the sensitivity of the capture ELISA, chromatographically-purified coproantigen was diluted in negative fecal supernatant and the dilutions were tested by capture ELISA. Using the OD value of the negative samples plus 2 SD, I was able to measure 300 pg of coproantigen per ml of fecal supernatant (Fig. 4.3).

Kinetics of coproantigen in the feces:

Fecal samples taken at 6, 8, 10, 13 week post-infection from 5 calves with high numbers of flukes were tested for coproantigen. A detectable level of coproantigen was present in

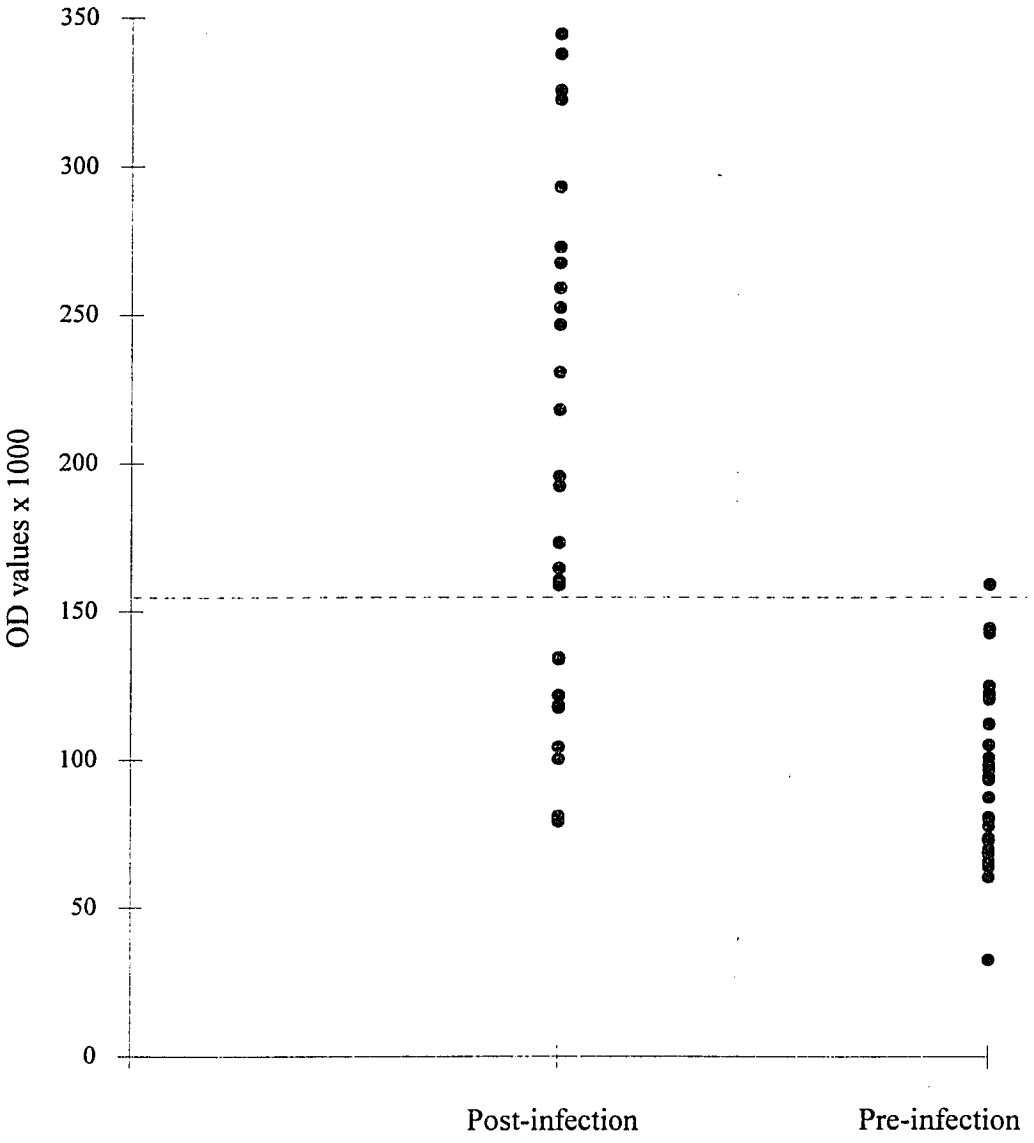

Fig. 4.1.Optical densities of capture ELISA from infected (n=27) and uninfected (n=27) animals. Dotted line represents mean OD of the negative animals + 2 SD.

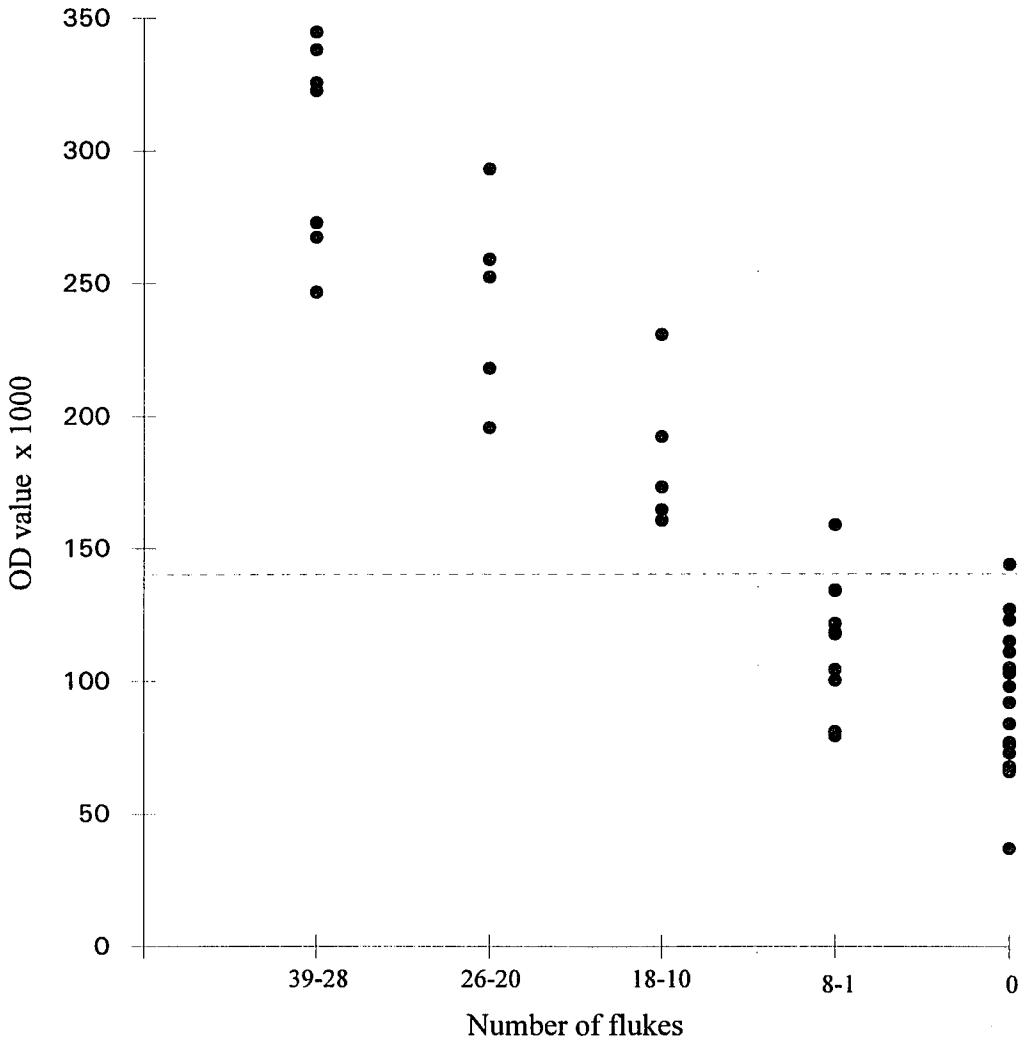

Fig. 4.2. Optical densities by capture ELISA of fecal samples from experimentally-infected calves (n=27) with different numbers of flukes.

Table. 4. 1. Representative triplicate results for capture ELISA for coproantigen illustrating inter-assay and intra-assay variation. Results of four assays (1, 2, 3, 4) preformed in four different days are shown.

ELISA OD from individual wells												
Test	39 fluke				36 Fluke				26 Fluke			
	A	B	C	mean	A	B	C	mean	A	B	C	mean
1	.232	.349	.356	.343	.329	.339	.311	.340	.347	.356	.259	.338
2	.436	.418	.429	.428	.394	.418	.422	.411	.364	.419	.402	.395
3	.378	.343	.369	.363	.370	.407	.416	.398	.362	.377	.386	.375
4	.386	.379	.402	.389	.375	.391	.362	.376	.362	.368	.331	.354

ELISA OD from individual wells (cont'd)												
Test	26 fluke				22 fluke				0 fluke			
	A	B	C	mean	A	B	C	mean	A	B	C	mean
1	.346	.346	.270	.275	.255	.294	.279	.275	.98	.94	.89	.94
2	.354	.401	.395	.383	.294	.380	.406	.360	.116	.98	.129	.112
3	.374	.354	.362	.363	.297	.376	.283	.319	.105	.95	.91	.97
4	.384	.353	.349	.362	.235	.364	.380	.326	.111	.100	.294	.276

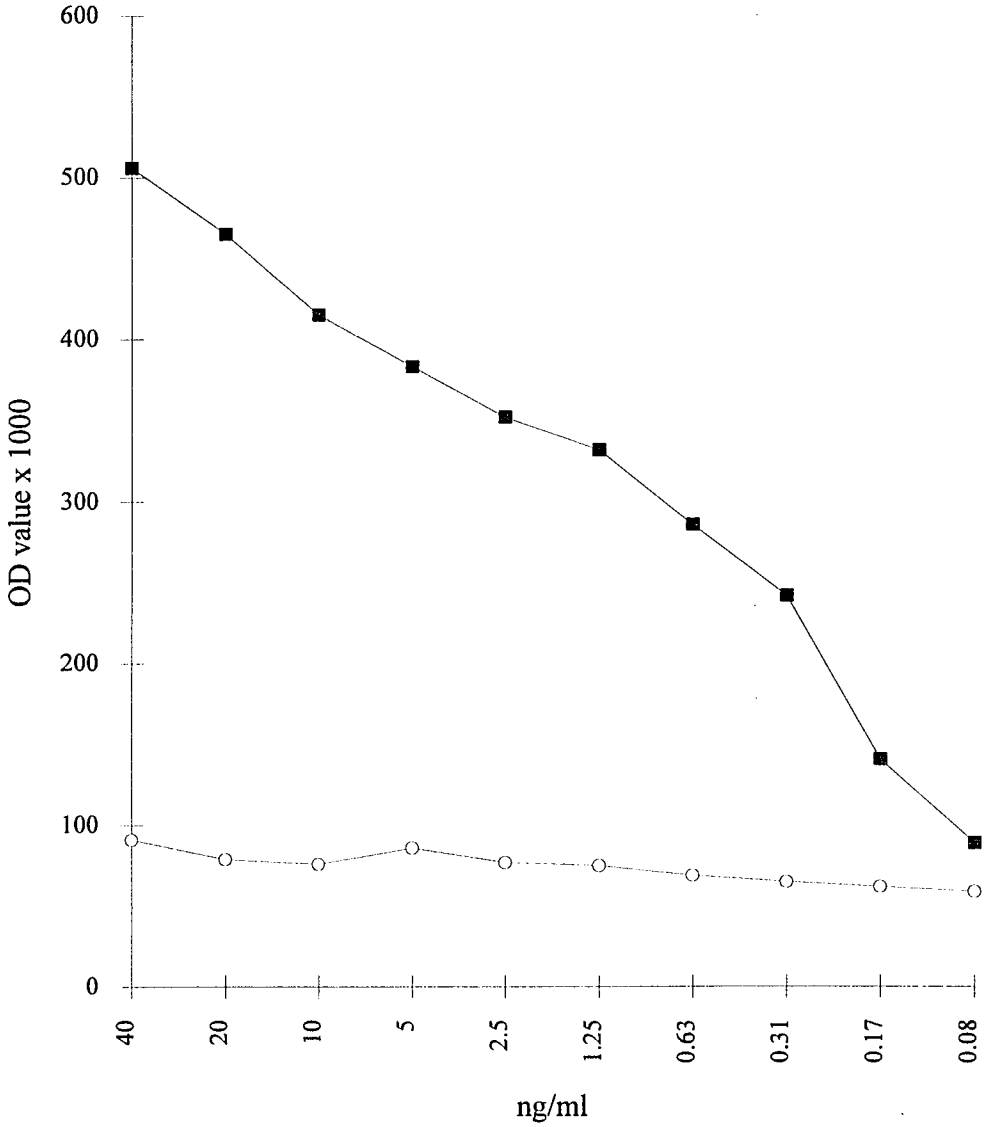

Fig. 4.3. Detection of purified coproantigen diluted in fecal supernatant from an uninfected calf: (■) purified coproantigen, (o) fecal supernatant from uninfected animals.

all five animals at 6 weeks post-infection. Maximum coproantigen levels occurred 8 weeks after infection. The coproantigen level remained constant until the end of the experiment (Fig. 4.4). No eggs were detected in the feces of the experimentally infected animals at week 6 and eggs were detected in the feces of only two of the animals 8 weeks (data not shown).

Relationship of coproantigen level, number of flukes and egg count:

Using the current procedure for determining fecal egg counts per 2 grams of feces, the egg counts for all the experimentally infected animals was low and detection of infection with less than 16 flukes was difficult. Of the 12 animals infected with less than 16 flukes only two had egg counts of 1 egg/2 gm of feces and the rest were negative. To increase the sensitivity of egg detection, 6 grams of feces were used in the sedimentation procedure and the egg counts ranged from 0 - 11 with a slight positive correlation with the number of flukes ($r = 0.39$). Capture ELISA results on the same fecal samples showed a strong correlation ($r = 0.96$) between the OD values of the fecal ELISA and number of flukes (Fig 4.5). There were also a slight correlation between the coproantigen and the egg count ($r = 0.36$; data not shown).

DISCUSSION

Definitive diagnosis of fasciolosis is usually achieved by the detection of eggs in the feces; however, this procedure is unable to predict the number of worms since the egg counts are variable and commonly less than 5 eggs per gram even in heavily infected herd. Moreover, herd egg counts peak and wane depending on seasonal transmission (Malone and Craig, 1990).

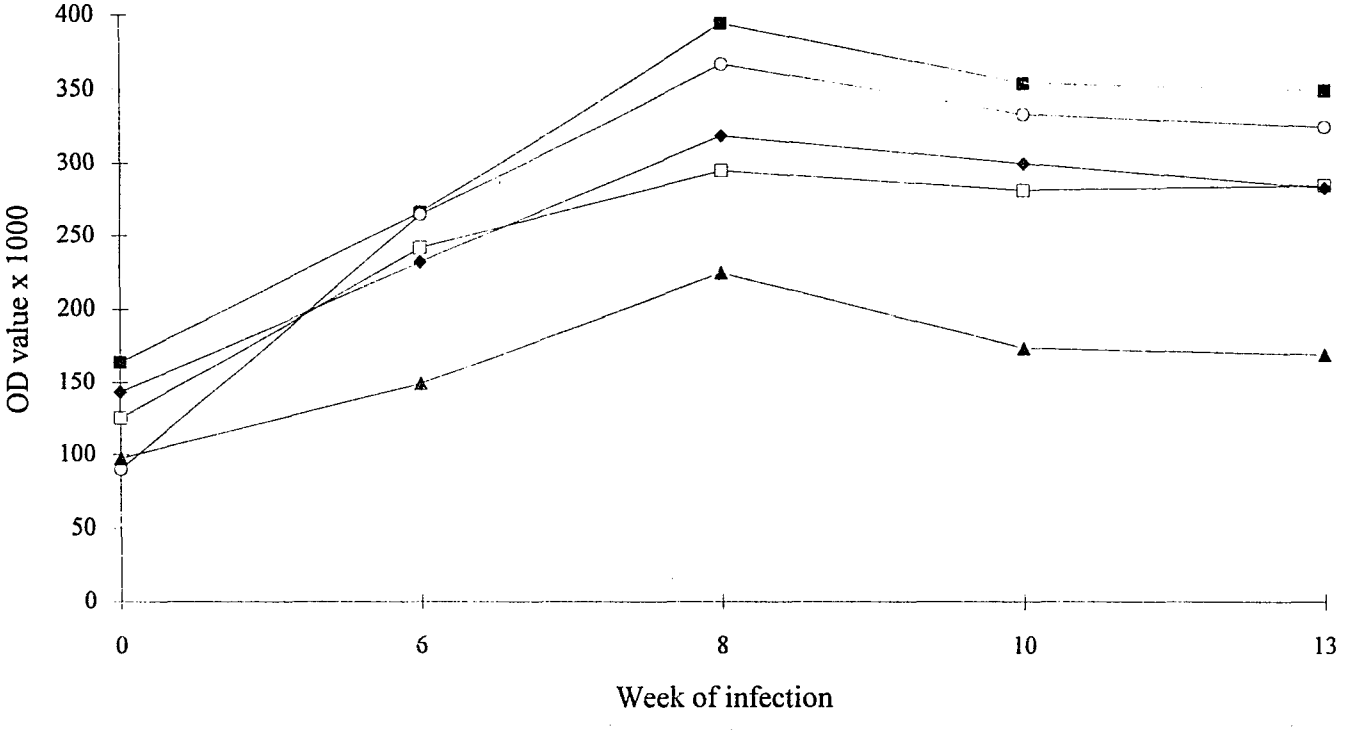

Fig. 4.4. Kinetics of coproantigen appearance in feces of experimentally infected calves with (■) 39 flukes, (□) 32 flukes, (○) 28 flukes, (◆) 22 flukes and (▲) 10 flukes.

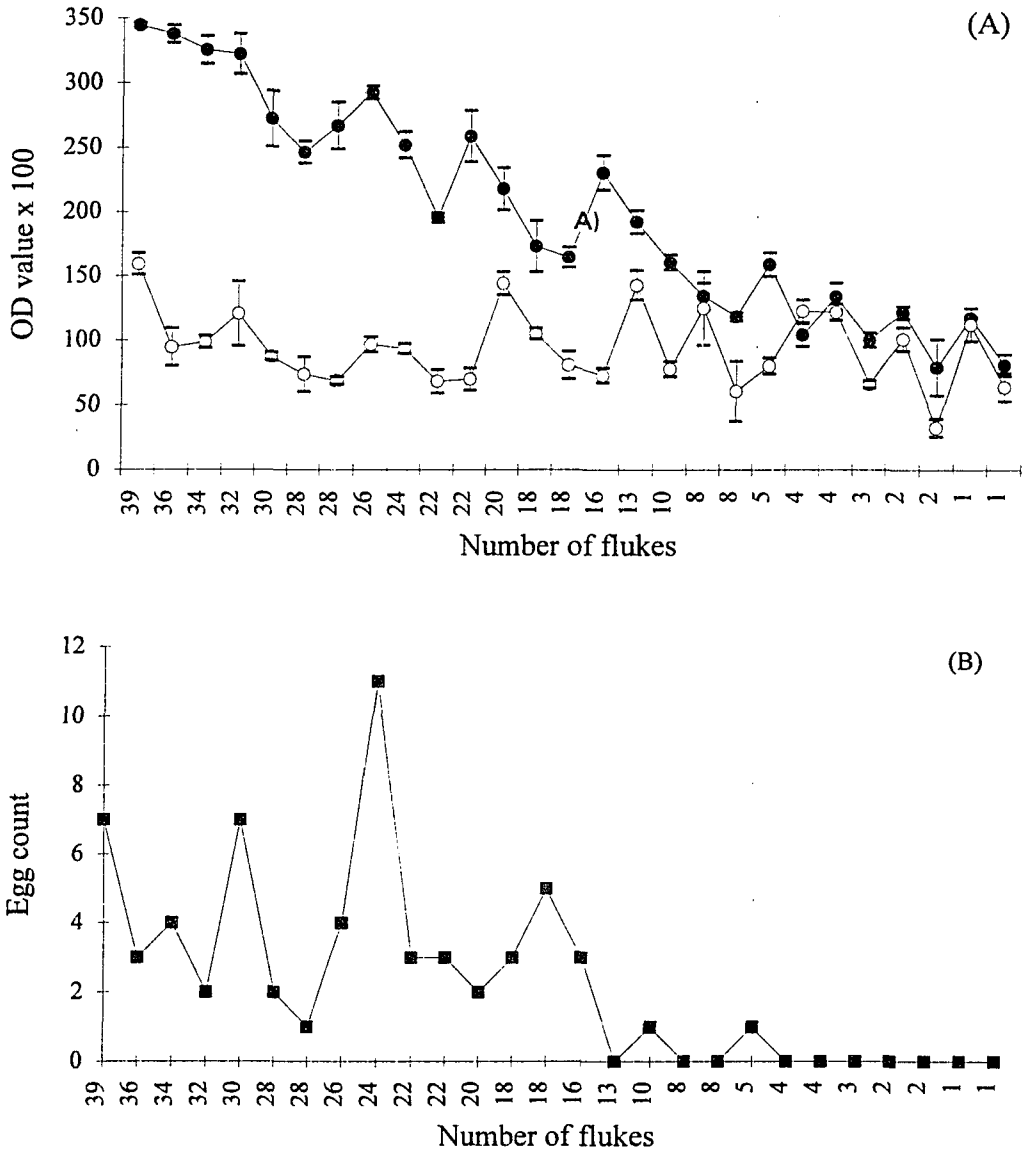

Fig. 4. 5. Correlation of the OD values of the capture ELISA and the egg per 2 gram of feces with the number of flukes. Panel (A) capture ELISA (r= 0.96) and panel (B) fecal egg count (r= 0.39); (●) Positive feces, (○) negative feces and (■) number of eggs.

In addition, *F. hepatica* eggs may be confused with other trematode eggs from parasites such as *Cotylophoron* sp. and *Paramphistomum* spp. Therefore immunodiagnostic assays may be superior for diagnosis of fasciolosis.

In this study, using a capture ELISA, I evaluated *Fasciola* infection in the experimentally-infected calves by detection of coproantigen. The sensitivity of the capture ELISA for the detection of infection with one or more flukes was 67% . I found that the ability of the test to detect coproantigen was correlated with the fluke burden (Fig 4.2). The highest absorbance values occurred with calves infected with 39-28 fluke and the absorbance decreased to median level for those calves infected with 26-20 and a low but detectable absorbance value was seen in animals infected with 18-10 flukes. The test could not differentiate between those animals infected with less than 8 flukes (n=10) and the uninfected animals. Samples from the animals with less than 10 flukes were combined with the negative samples (n=17) and these were the negative control used to determine the sensitivity of the test for detection of infections with 10 flukes or more. The sensitivity of detection of 10 flukes or more was 100% with specificity of 96.3%.

I previously localized the 26-28 kD coproantigen that binds to the MAb F10 to the tegumental cell and in the gut lining of the bile duct stages flukes (Chapter III). In the present study, the coproantigen was detected by the 6th week after infection. This coincides with the arrival of the majority of the flukes in the bile duct (Soulsby, 1968) and when secretory and excretory products would be secreted into the bile. These results are similar to those obtained by WB (Chapter II). Samples prior to 6 weeks post-infection were not available,

therefore I do not know if the antigen is detectable before 6 weeks. Based on previous reports, I believe that the antigen would not be detectable prior to 6 weeks since the flukes are outside the bile ducts, thus the antigens would not be shed into the feces but would probably be present in the circulation. Maximum detection of the coproantigen occurred at 8 weeks post-infection. This could be attributed to the rapid growth and maturation of the flukes which would be associated with a higher metabolic rate and increased shedding of the glycocalyx. I observed a very slight decrease in the absorbance values at 10 week after infection after which the level of coproantigen remained stable until the 13 week post-infection, when the experiment was terminated. My results complement those obtained by Langley and Hillyer (1989b) and by Rodriguez- Pérez and Hillyer (1995). In their studies they observed a marked decrease in the level of the circulating immunecomplex and the circulating antigen by week 10 post-infection, which they attributed to the establishment of the parasite in the bile duct and the availability of relatively less antigen in the circulation. Therefore, when the antigen decreases in the circulation after establishment of the fluke in the bile duct, it becomes detectable in the feces. After establishment in the bile duct there is continuous shedding of the surface antigens but at a slightly slower rate (Hanna et al., 1980b) This slower rate of shedding is reflected by the slight decrease in the absorbance value at 10 weeks post-infection. This is an advantage of the capture ELISA for coproantigen over other immunoassays.

I determined that the capture ELISA for coproantigen was sensitive enough to detect 300 pg coproantigen/ ml of fecal supernatant. This is 80-fold more sensitive than the ELISA for circulating antigen (25 ng/ml) reported by Langley and Hillyer (1989a) and 50-fold more

sensitive than the 15 ng/ml that reported by the reported by Espino et al. (1994). The high sensitivity of this assay facilitates detection of low fluke burdens where the antigen is highly diluted in large amount of ingesta, as in the case of cattle.

Malone and Craig (1990), reported that yearling calves infected with less than 20 flukes shed an average of 1-5 eggs per 2 gram feces, those infected with 30 flukes shed an average of 5-10 eggs and those who infected with 60 fluke shed an average of 10-40 eggs per two grams of feces. In this study I could not detect eggs in 2 grams of feces from infected calves at 6 or 8 weeks after infection. At 10 weeks eggs were detected in only 2 animals infected with less than 13 flukes . When 6 gm of feces were used in the sedimentation procedure to increase the sensitivity of egg detection, I found two of the animals were shedding eggs 8 weeks post-infection. The correlation between the fluke burden and the egg count ($r = 0.39$) was low compared to the strong correlation between the coproantigen and the number of flukes ($r = 0.96$). Duwel and Reisenleiter (1990) monitored egg shedding in bulls experimentally infected with different numbers of *F. hepatica* metacercaria and found that *Fasciola* egg counts fluctuated within one animal and within one infection group. He also found that the distribution of *Fasciola* eggs in the feces is irregular within one day and also over several days in all animals. In contrast, coproantigen detection is relatively stable as long as the flukes are in the bile duct.

Capture ELISA for coproantigen provides a sensitive, specific and rapid procedure for diagnosis of fasciolosis. The assay correlated well with the number of flukes making it possible to estimate fluke burden. I was able to detect early infection, that is before the

adults mature and start to shed eggs. In addition, the capture ELISA has distinct advantages over other assays: 1) it only detects the antigen as long as the parasite is present in the bile duct, and 2) the simplicity of the sample collection, 3) Potential use of coproantigen detection for other mammalian tests of *F. hepatica*, including human infections .

CHAPTER V

CONCLUSIONS

The presence of parasite specific antigens in the feces of infected hosts has recently received considerable attention as a means of diagnosis of a number of parasites. The ultimate goal of this work was to develop a rapid, sensitive and specific test for detection of *Fasciola hepatica* coproantigen using MAbs. The specific aims were: 1) to determine the sensitivity and the specificity of the available 4 MAbs to the 26-28 kD antigen present in the feces of infected cattle using WB, 2) to characterize the antigen containing the epitope recognized by these MAbs; 3) to localize the coproantigen on the bile duct stages of liver flukes and 4) to develop a capture ELISA for detection of the coproantigen.

My first aim was determine the specificity and the sensitivity of the available MAbs to the coproantigen. Western blot analysis was used to detect the presence of coproantigen in feces of experimentally infected calves. Using WB and the colorimetric substrate TMB, culture supernatant containing the 4 MAbs were shown to react with a 26-28 kD band of the excretory secretory product (ES) but the MAbs were not concentrated enough to reveal the band in the feces. However, the band was detected in the feces using ECL substrate which indicated that ECL is more sensitive substrate than TMB. By using purified MAbs at 10 μg/ml, the 26-28 kD coproantigen could be detected in the feces of infected animals. To further enhance the detection, a biotin-streptavidin modification was employed and was found to increase the sensitivity. I found that the most sensitive detection of the coproantigen by

WB (>10 flukes) was achieved using the biotin-streptavidin modification and TMB substrate. To determine the specificity of the MAbs for *F. hepatica*, the MAbs were then tested against fresh fluke antigen (FAA) and ES of other trematode and cestode parasites that commonly occur in cattle or that are related to *F. hepatica*. For example, *Paramphistomum* spp. eggs are sometimes confused with *F. hepatica* eggs in routine fecal sedimentation examinations. Using WB, I found that the MAbs do not recognize antigens in the ES of *P. microbothriodes,* an advantage of the test. There was also no cross-reactivity with the cestode parasite *Moniezia sp*. However, minimum cross-reactivity was observed with ES of *F. gigantica,* which is found in tropical and subtropical regions of the world and therefore would not be a problem with samples in North America. The MAbs also cross-reacted with the ES of *Fascioloides magna*, a deer liver fluke that sometimes incidentally infects cattle in ranges shared with deer. However, cattle are an abnormal host, and encapsulation of *F. magna* typically prevents shedding of eggs into the feces. Further investigation is needed to determine if the *F. magna* antigens are excreted in the feces of infected animals and whether these antigens can be detected in the feces by these MAbs.

The second aim of this study was to characterize the nature of this antigen. The 26-28 kD antigen was purified from ES by gel filtration. The purified 26-28 kD antigen was unchanged under reducing and non-reducing conditions. The pattern of staining of the 26-28 kD coproantigen with differential stains suggested that the molecule is a proteoglycan. Alkaline treatment of the purified coproantigen resulted in the disappearance of the 26-28 kD band and generation of a new band of 8 kD that was recognized by the MAbs in WB.

Endoglycosidase F or endoglycosidase H had no effect on the coproantigen. Together, these results indicated that the coproantigen is a monomeric proteoglycan of which 70% of its mass is a carbohydrate and is probably O-glycosylated. Further, the specific epitope recognized by the MAbs is on the protein core, not in the carbohydrate component. Potential protease activity of the coproantigen, as determined by gelatin SDS-PAGE, indicated that the coproantigen is not a protease.

In transit from the bile ducts through the intestinal tract, the coproantigen is exposed to digestive enzymes such as pepsin (found in the gastric juice) and trypsin (found in the small intestinal digestive fluid) and is therefore exposed to the effect of these proteases. The antigenicity of the coproantigen was not affected by the treatment by pepsin, but treatment with trypsin resulted in the generation of an 18 kD molecule which retained the epitope recognized by the MAbs used in this study. Studies on the effect of the different storage temperatures on the coproantigen revealed that although there are shifts in the band size due to an apparent gradual loss of glycosylation or proteolytic cleavage, the antigenicity was retained.

The third specific aim was to localize the antigen on the flukes. Three of the MAbs (F10, G7 and E7) were found to recognize an antigen in the tegument and the gut of the adult worm. The fourth MAb (F7), recognized antigen only in the gut of the fluke. The resultswere identical for immature bile duct flukes.

The fourth specific aim was to develop a sensitive, rapid and easy test for diagnosis of fasciolosis. I developed a capture ELISA to detect the coproantigen in calves experimentally-

infected with *F. hepatica*. The assay was able to detect *Fasciola* in animals infected with more than 10 flukes. I found that the ability of the test to detect coproantigen was positively correlated with the fluke burden ($r = 0.96$) and that the test was able to detect as little as 300 pg of coproantigen per ml of fecal supernatant. Detectable level of coproantigen was present in all five animals at 6 weeks post-infection and that maximal coproantigen levels occurred 8 weeks post-infection. However, no eggs were detected in the feces of the experimentally infected animals at week 6 and only two of the had animals started egg shedding at 8 week. Together, these data indicate that this capture ELISA for the *F. hepatica* 26-28 kD coproantigen is a more sensitive and quantitative assay than fecal egg counts. In addition, the assay is rapid, easy to perform and lends itself well to large numbers of samples. The test may be useful for detecting infections on other hosts, including zoonotic human infections. The assay can detect infection as early as 6 weeks post-infection, before flukes are sexually mature and producing eggs.

Future studies:

Future studies will concentrate on field evaluation of the sensitivity and the specificity of this test on cattle naturally infected with *F. hepatica*. Since the earliest time point in my study was 6 weeks post infection, additional experimental-infections are needed to determine how soon after infection the coproantigen can be detected. These studies could also address the possibility that the 26-28 kD antigen may be present in the circulation prior to the arrival of the flukes in the bile duct.

Capture ELISA may also be useful in the detection of human fasciolosis. Since this capture ELISA is very sensitive and since human feces are not as diluted as the feces of cattle, it might be possible to detect a single worm infection in human patients using the capture ELISA. The possible cross-reactivity with other human parasites should also be examined.

The potential usefulness of the coproantigen as a vaccine could be tested by immunization of rats with the chromatographically purified 26-28 kD antigen followed by challenge with *F. hepatica* metacercarae. If the 26-28 kD antigen was protective, the protein could be sequenced. DNA primers deduced from the protein sequence would allow the gene to be identified. This would supply additional information on the biology of the coproantigen as well as act as a step toward the development of a recombinant vaccine against *F. hepatica* infection.

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Major Field: Veterinary Medical Sciences

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